UNIVERSITY OF ALBERTA

# ASCORBIC ACID SUPPLEMENTATION TO DAIRY CATTLE

BY

DARREN MACLEOD ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

IN

ANIMAL NUTRITION

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## ABSTRACT

Ruminant animals are capable of endogenously producing sufficient quantities of ascorbic acid to meet their metabolic requirements under normal physiological conditions. Stress or high levels of production may predispose dairy cows to subclinical deficiencies, therefore, experiments were conducted to investigate the role of vitamin A.

The objectives of these studies were: 1) examination of the effect of abomasal infusion of ascorbic acid on circulating levels of plasma cortisol in lactating dairy cows and 2) evaluation of ascorbyl-2-polyphosphate as a possible source of rumen stable ascorbic acid for ruminants.

Four rumen fistulated, lactating, Holstein freisen cows were used in a 4x4 latin square design to assess if graded levels of ascorbic acid infused into the abomasum would influence plasma cortisol. Plasma cortisol and ascorbic acid levels were not significantly altered ($P>0.05$), although there was a trend indicating that plasma levels of ascorbic acid were increased.

Ascorbyl-2-polyphosphate and crystalline ascorbic acid were incubated in the rumen fluid of four dry Holstein freisen cows. The half lives were 3.46 and 5.21 hours for the crystalline ascorbic acid and the ascorbyl-2-polyphosphate respectively, indicating that the polyphosphate form has greater rumen stability.

Ascorbic acid when injected into the jugular vein of four dairy cows distributed itself according to a two compartment open model. The terminal half-life was determined to be 11.4 hours.

A method to isolate purified bovine polymorphonuclear leukocytes was developed. The procedure yielded highly purified viable cells, 97.6% $\pm$ 1, polymorphonuclear leukocytes.

Ascorbyl-2-polyphosphate was fed to six Holstein freisen dairy heifers for 31 days at a rate of 80 grams per day (20 grams ascorbic acid equivalent). The plasma levels of the ascorbyl-2-polyphosphate treated heifers was significantly higher than the controls, 4.56 versus 3.58 mg/L respectively. Ascorbic acid content of the biceps femoris indicated a definite trend towards higher ascorbic acid levels in the muscle of the treated animals (P=.08). The ascorbyl-2-polyphosphate did not alter the ascorbic acid content of the neutrophils from the treated heifers.

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# CHAPTER 1.

# INTRODUCTION

## 1.1 ASCORBIC ACID

### 1.1.1 CHEMISTRY

An overview of the chemistry of ascorbic acid is essential for an understanding of the functions of this compound in biological systems. Ascorbic acid is a simple compound with a molecular weight of 176.1 and a structural formula of $C_6H_6O_6$. The ionization of ascorbic acid occurs in two stages: 1) the first stage has a pK value of 4.2 at 37°C and occurs at C-2 and 2) the second stage occurs at C-3 at a pK value of 11.6 (Levine and Morita, 1985). At normal physiological pH the compound is therefore almost entirely in the anionic form.

Ascorbic acid plays a critical role in biological reactions because it has the

ability to donate electrons while undergoing reversible oxidation to dehydroascorbic acid (Bielski et al., 1975). There is an intermediate oxidation product that occurs before the formation of dehydroascorbic acid, it is called the ascorbate free radical or semidehydroascorbate.

The dehydroascorbate/ascorbate redox pair are in equilibrium but are influenced by light, pH and possibly temperature. At pH 7.0, physiological pH, the dehydroascorbate molecule is very unstable and is rapidly hydrolysed to diketogulonic acid (Tolbert and Ward, 1982). Diketogulonic acid is an irreversibly oxidized product of dehydroascorbate and no longer possesses antiscorbutic properties. Dehydroascorbate is then further oxidized to L-threonic and carbon dioxide or L-threonic acid and oxalic acid (Hornig et al., 1984).

### 1.1.2. BIOSYNTHESIS

The ability to synthesize ascorbic acid is inherent to most domestic animals. In evolutionary terms invertebrates and fish did not possess the capability to endogenously produce ascorbic acid but as life on earth evolved from the aquatic to the terrestrial, the emergence of species, particularly amphibians that had the ability to produce ascorbic acid arose. Initially, ascorbic acid was produced only in the kidneys of reptiles, birds and amphibians but with the evolution of marsupials the synthesis began occurring in the liver and kidney. At present, the vast majority of mammals that are capable of synthesizing ascorbic acid do so exclusively in the

liver. There is extreme variability between and amongst species with respect to the ability of the liver microsomes to produce ascorbic acid (Chatterjee, 1973).

Ascorbic acid is synthesized from D-glucose or galactose following the glucouronic acid pathway. L-gulonic acid lactonizes to L-gulono-γ-lactone and is oxidized by the key enzyme which is missing in animals incapable of synthesizing ascorbic acid, L-gulono-γ-lactone oxidase (Chatterjee et al., 1975; King, 1973). Sato (1980) in an elegant study confirming this hypothesis implanted dialysis bags containing L-gulonolactone and L-gulonolactone oxidase into guinea pigs and fed the animals a vitamin C devoid diet. The animals implanted with the precursors for vitamin C did not show any clinical signs of scurvy while the control animals did.

### 1.1.3. ABSORPTION AND DISTRIBUTION

It appears the animals incapable of synthesizing ascorbic acid have a different mechanism for absorbing this compound than do animals capable of endogenous production. In man and guinea pigs ascorbic acid is absorbed actively via a sodium energy dependent pump. The Km value for the transport of ascorbate into the brush border of guinea pigs and man is 0.3 and 0.5 mM, respectively (Hornig et al., 1984). Guinea pigs absorb ascorbic acid primarily in the duodenum while rats absorb the vitamin passively in the ileum (Hornig et al., 1984).

In most species of animals the highest concentration of ascorbic acid occurs in the endocrine glands. Adrenal and pituitary tissue can contain 200-fold the

amount of ascorbic acid found in blood (Hornig, 1975). The major difference between species that can produce ascorbic acid and those that can not is that tissue levels in animals that do produce ascorbic acid are appreciably higher. Organs such as the brain, liver, kidney and heart muscle contain intermediate levels of the vitamin in the range found between that in plasma and the endocrine glands (Hornig, 1975). Polymorphonuclear leukocytes have a higher level of ascorbic acid than plasma, an approximate 75 fold difference in humans (Kutnink et al., 1987). In human peripheral mononuclear leukocytes the magnitude of ascorbic acid within the cells was 100 fold greater than plasma (Bergsten et al., 1990).

### 1.1.4. FUNCTIONS OF ASCORBIC ACID

#### 1.1.4.1. COLLAGEN FORMATION

Ascorbic acid is involved in many cellular biochemical processes due to its ability to donate electrons and then be reduced to its functional form once more. The exact role that vitamin C plays in metabolism has never been precisely defined because as of yet a coenzyme has not been reported.

The most established and documented role of ascorbic acid is in collagen synthesis. Vitamin C is an essential factor in the hydroxylation of peptide bound proline and lysine, which are essential steps in the production of procollagen (Chatterjee, 1989). The procollagen fibrils are then cross-linked with the participation of lysyl, a pyridoxine dependent enzyme (Weiser et al., 1992).

### 1.1.4.2. VITAMIN D METABOLISM

Vitamin C is also required in the hydroxylation of cholecalciferol into the biologically active forms 25-hydroxycholecalciferol and 1,25-dihydroxycholecalciferol. This is extremely important for domestic animals and humans alike as the commercially available vitamin D supplements are cholecalciferol and without further hydroxylations in the liver and kidneys are of little or no use to the animal (Sergeev et al., 1990). Vitamin D in the hydroxylated form acts very much like a hormone and can regulate the phosphorous/calcium homeostasis of animals via its influence on calcium binding protein and parathyroid hormone (Weiser et al., 1992). Ascorbic acid acts as a reducing agent in these reactions but it has of yet to be proven if it could be replaced by other reducing agents such as dithiotheitol. Dithiotheitol is a potent reducing agent and is often used in the preparation of biological samples containing ascorbic acid to preserve the compound by stopping the oxidation to dehydroascorbic acid (De Antonis, 1993).

## 1.2. ASCORBIC ACID ANALYSIS

Ascorbic acid is a very difficult compound to analyse regardless which technique is used. In biological samples that require extensive sample preparation

there is always a real danger that ascorbic acid will be oxidized before the analysis is conducted. It therefore is necessary to process samples as quickly as possible after collection and even then problems arise with sensitivity, specificity and substance interference. It is critical to be able to measure ascorbic acid precisely and accurately if animal requirements or kinetics are to be examined.

### 1.2.1. CHEMICAL ASSAYS

Assays of a chemical nature are primarily based on the oxidation of ascorbic acid. The most common chemicals used are bromine, iodine, 2,6-dichlorophenol-indophenol and ferric salts. The most frequently used method is one using 2,6-dichlorophenol-indophenol as the indicator dye. Ascorbic acid reduces the dye causing reduced absorption at 520 nm (Cooke and Moxon, 1981). The procedure is typically run at a pH of 3.0-4.5 under nitrogen to minimize interference from oxidized forms of the ascorbate, however, this method is not without serious limitations, particularly with regards to analyses of animal tissues and fluids where specificity is a concern. This protocol is not highly specific because the dye can be reduced by other compounds such as reductones, sulfhydryl compounds, phenols and thiols (common contaminants of tissue/fluid samples) (Cooke and Moxon, 1981). Ferric iron and copper II can also interfere with the analysis by oxidizing the ascorbic acid prior to analysis. A further complicating factor is one of poor

sensitivity. The sensitivity of this procedure is 34 nmol/sample which limits its usefulness when analyzing ascorbic acid concentrations on the cellular level (Washko et al., 1992).

The ferrous chromogenic complex method of analysis also depends on the reducing ability of the ascorbic acid. Ferric iron is reduced to ferrous iron by ascorbic acid, the ferrous iron is then reacted with a chelating agent forming a coloured complex that can be measured spectrophotometrically (Cooke and Moxon, 1981). The most often used chelating agents are 2,2-dipyridyl and ferrozine (McGown et al., 1982). McGown and coworkers (1982) came to the conclusion that the sensitivity was 400 pmol/sample and therefore did not have practical application. As with the previous reductive assay, the specificity of this test is poor due to interference from reducing agents such as alpha-tocopherol, glutathione and cysteine which may also have the ability to chelate iron.

### 1.2.2. GAS-LIQUID CHROMATOGRAPHY

To analyse for ascorbic acid by gas chromatography the compound must be derivatized with trimethylsilyl ether and be completely free of water (Cook and Moxon, 1981). Ascorbic acid reacts to form a tetratrimethylsilyl ether and must be maintained absolutely free of water to avoid hydrolysis and reduced activity (Washko et al., 1992). The time required for the derivatization of the ascorbic acid in biological samples may take as long as 24 hrs making this method very

impracticle. The sensitivity is also not as good as other methods being in the range of 280 pmol/sample (Washko et al., 1992).

### 1.2.3. ENZYMATIC PREPARATION

Ascorbic acid oxidase has been used successfully in conjunction with a chemical treatment to analyse for ascorbic acid (Liu et al., 1982). Identical samples with and without ascorbic acid were incubated with ascorbic acid oxidase, ferric iron and 2,4,6-tris(2-pyridyl)-5-triazine. The absorbance was then read at 593 nm; the difference in the replicate samples was proportional to the ascorbic acid level (Liu et al., 1982). The major problem with this analytical technique is that there is no way to measure for dehydroascorbic acid.

### 1.2.4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Sensitivity and interference problems with the previously mentioned methods of analyses has lead to the development of a high performance liquid chromatographic technique. In past studies that examined biological materials, levels were often over estimated due to the presence of other compounds containing antioxidant properties. This is an extreme liability when examining the levels of ascorbic acid in plasma or leukocytes of animals and humans, often leading to inconclusive or misleading research results.

### 1.2.4.1. ULTRAVIOLET DETECTION

High performance liquid chromatography has allowed scientists to measure ascorbic acid in a very sensitive and accurate way. One of the earliest methods developed using high performance liquid chromatography and ultraviolet detection was conducted by Rose and Nahrwold (1981). In these studies the absorbance was set at 254 nm, subsequent papers have shown that at this wave length absorbance is approximately 80% of the maximum (Finley and Duang, 1981). However, mercury lamp fixed wave length detectors are inexpensive and reliable and will probably continue to be the detector of choice for the determination of ascorbic acid. Finley and Duang (1981) also reported the concurrent separation of ascorbic acid and dehydroascorbic acid. Two reversed-phase columns in series with a mobile water phase containing an ion-pair reagent and utilising two detectors set at 254 nm and 210 nm for ascorbic acid and dehydroascorbic acid, respectively., was used. The separation techniques which appear to work well are ion-pair reverse phase and ion exchange chromatography (Charles et al., 1983; Wagner et al., 1979; Pachla and Kissinger, 1976).

High performance liquid chromatography with ultraviolet detection is more specific and has greater sensitivity than previously mentioned techniques. Sensitivity is normally in the range of 50 pmol/injected sample (Kutnink et al., 1987). Ascorbic acid samples are frequently stabilized with metaphosphoric acid dithiotheitol (Nagy and Degrell, 1989). In order to protect the ascorbic acid from

prooxidant minerals such as ferric iron and copper, chelation agents such as EDTA are also used (Dhariwal et al., 1990). Antioxidant amino acids such as cysteine and homocysteine have also been used, however, the results are variable and these compounds do not tend to be used in most laboratories (Bates, 1992).

Antioxidants do present a few additional sources of error that must be taken into account. For example, dithiotheitol will convert any dehydroascorbic acid in the sample to ascorbic acid which may give higher analysed levels of ascorbic acid. Dithriothreitol will also absorb at 254 nm making it unsuitable for ultraviolet detection. It is usually accepted that despite the shortcomings of antioxidants, they are critical in terms of sample stabilization.

High performance liquid chromatography with ultraviolet detection is a very reliable method for ascorbic acid analysis. In some biological samples, particularly with small sample sizes or where levels between the treated and control are small, the detection limit may not be adequate. Another draw back to the aforementioned method is there is no method available to analyse for dehydroascorbic acid directly.

### 1.2.4.2. FLUORESCENCE DETECTION

In an attempt to analyse for both ascorbic acid and dehydroascorbic acid a high performance liquid chromatographic fluorescence technique was developed (Iwata et al., 1985). The dehydroascorbic acid in the sample was reacted with 1,2-diamino-4,5-dimethoxybenzene to produce a highly fluorescent product (Iwata et

al., 1985). Although fluorescence detection is very sensitive and can analyse for both the ascorbic acid and dehydroascorbic acid, sample preparation time is long and labourious. This technique is therefore not practical when dealing with a large number of samples as is often the case in animal research.

### 1.2.4.3 ELECTROCHEMICAL DETECTION

Vitamin C is a compound that is very easily oxidized and therefore is electrochemically active. The advent of high performance liquid chromatography had made this method of detection possible because other electro active substances in biological fluids can now be effectively separated out (Pachla and Kissinger, 1976). Concurrent with the development of high performance liquid chromatographic techniques, thin layered amperometry was also introduced (Pachla and Kissinger, 1976). In principle, electrochemical detection is dependent on the oxidation or reduction of a solute and the subsequent quantification of the solute based current. The mobile phase must allow for distinct separation of the ascorbic acid and be conductive enough to carry the charge while maintaining only a small amount of background noise. The background noise is extremely critical, as any stray outside voltage arising from other equipment or static electricity from the operator, will lead to a very noisy base line and greatly diminish the sensitivity of the test.

The majority of high performance liquid chromatography techniques for analysing ascorbic acid utilize amperometric detection. A working glassy carbon electrode is set at .6 -.8 volts against an Ag/AgCl reference electrode (Nagy and Degrell, 1989). It is critical to keep the glassy carbon electrode polished and free of contaminants or sensitivity is greatly reduced. The common columns to use are very similar to the type used in ultraviolet detection, namely reverse phase ion exchange and ion paired reverse phase (Dhariwal et al., 1990).

Ascorbic acid is commonly extracted from tissues and fluids with metaphosphoric acid for electrochemical detection (Kutnink et al., 1987). In addition, antioxidants such as homocysteine dithiotheitol are often added to the metaphosphoric acid for additional protection (Kutnink et al., 1987).

To protect the ascorbic acid from prooxidative minerals such as ferric iron and copper, EDTA can be included in the metaphosphoric extraction solution (Kutnink et al., 1987; Nagy and Degrell, 1989). When plasma or cerebrospinal fluid was preserved with metaphosphoric acid and EDTA it was stable for 10 hours at 4°C and 6 hours at room temperature (Kutnink et al., 1987; Nagy and Degrell, 1989). The detection limit for this type of assay is normally 10 pmol per injection sample (Washko et al., 1992). The detection limit can be considerably higher depending on the strength of the metaphosphoric acid used to stabilize the sample. Metaphosphoric acid has a high ionic strength and can reduce the sensitivity of the test but this is not normally a concern when looking at plasma, tissue or large

numbers of neutrophils.

### 1.2.4.4. COULOMETRIC DETECTION

Amperometric detection differs from coulometric detection in that amperometric readings are determined by the concentration of the solute while coulometric detection is mass dependent. In addition, most electrochemical detectors are, flow by, where the electrode is embedded in the side of the detection cell. The surface area of the electrode is small compared to that of the detection cell. Coulometric detectors have flow through porous graphite detectors that the solvent passes directly through and thereby comes in contact with a large proportion of the surface area of the electrode. This enables this measurement technique to be extremely sensitive as much less solute is required (Dhariwal et al., 1990). Coulometric detection can easily measure less than 10 pmol per sample injected. However, it is even more prone to stray voltages and contaminants of electrochemical nature than amperometric detection. Dehydroascorbic acid can not be measured directly with this type of analysis. It is an expensive piece of equipment and will probably not be in routine use at labs which are analysing for ascorbic acid.

## 1.3. ASCORBIC ACID AND IMMUNITY

Morbidity and mortality in domestic animals is primarily caused by infectious disease. Therefore, it is extremely important to maximize the inherent antimicrobial defense mechanisms of the animal if production/profitability is to be maximized. Antioxidant systems within the animal, in which ascorbic acid is essential, play a key role in immunomodulation.

### 1.3.1. ANTIOXIDANT SYSTEMS IN THE ANIMAL

Free radicals and reactive oxygen species have long been known to have negative impact on the disease fighting capabilities of man and animals (Frei, 1994). It is therefore necessary to have an understanding of the antioxidant protection systems of the animals.

#### 1.3.1.1 LIPOPHILIC ANTIOXIDANTS

Vitamin E is the major lipid soluble antioxidant in the animal. It is a highly lipid soluble compound and is found in cellular membranes and membranes of the endoplasmic reticulum and mitochondria (Oski, 1980). Vitamin E protects membranes by scavenging peroxyl and alkoxyl radicals and preventing further peroxidative damage. Alpha-tocopherol is essential for maintaining the membrane

integrity of the immune, cardiovascular and nervous systems (Nockels, 1979).

β-carotene and other carotenoids including vitamin A are also lipid soluble. β-carotene protects cellular membrane integrity by scavenging reactive oxygen species and free radicals (Burton and Ingold, 1984). In conjunction with vitamin E the carotenoids form a protective mechanism inside cellular membranes to protect these from oxidative damage.

### 1.3.1.2. ENZYMATIC ANTIOXIDANTS

There has been a great deal of research conducted on the role of trace minerals in animal health and productivity in recent years. The trace mineral requirements of domestic animals are extremely critical if the animal is to reproduce and grow effectively. Part of the explanation for the importance of trace minerals is their critical role as co-factors in enzymatic antioxidant protection of cells and organs.

Superoxide dismutase consists of three different enzymes with the trace mineral co-factors, zinc, manganese and copper. In plasma and cytosol the predominant forms of superoxide dismutase is found primarily within the mitochondria. Superoxide dismutase protects membranes by converting the superoxide radical to hydrogen peroxide which is then converted to water by the enzymes catalase and glutathione peroxidase.

The enzymes catalase and glutathione peroxidase have as their co-factors

iron and selenium. Both enzymes do ostensibly the same tasks in the cytosol, that is convert hydrogen peroxide and reactive oxygen species to water. It is thought, however, that under conditions of disease or inflammation that catalase is the predominant enzyme while glutathione peroxidase is critical during everyday metabolic cellular processes (Jones et al., 1981).

### 1.3.1.3. HYDROPHILIC ANTIOXIDANTS

Ascorbic acid is a cytosolic, hydrophilic compound that can react directly with superoxide and hydroxyl radicals. Ascorbic acid was also shown by Frei and coworkers (1989) to be an excellent antioxidant in plasma where it reduced the peroxidation of plasma lipids. Ascorbic acid has also been shown to act synergistically with alpha-tocopherol in that ascorbic acid can reductively regenerate the active vitamin E molecule from the vitamin E radical (Niki, 1987).

Ascorbic acid, not unlike other antioxidants can have a prooxidant effect if it is present in concentrations greater than the physiological norm. Vitamin C may sometimes reduce ferric iron to ferrous iron which can generate free radicals and have a prooxidative effect (Hallberg et al., 1987). Copper and ascorbate may react in a similar way producing free radicals and a prooxidative effect.

Bendich et al. (1983) in an elegant study compared weight gain and T and B cells mitogen responses in guinea pigs fed vitamins C and E in various combinations and found that the greatest weight gain and immune responses

occurred when the animals were fed both vitamins simultaneously.

Also located within the cytosol of cells is a tripeptide, glutathione. Glutathione can act independently as an antioxidant or in conjunction with selenium as part of the enzyme glutathione peroxidase. Ascorbic acid and glutathione can also work synergistically in that glutathione can reduce dehydroascorbic acid back to its biologically active form, reduced ascorbic acid.

### 1.3.2. ASCORBIC ACID AND NEUTROPHIL FUNCTIONALITY

Neutrophils are the main component of the immune response which help dairy cattle stave off clinical or sub-clinical mastitis. Mastitis in its many forms is a chronic problem on modern dairy farms. In the United States alone mastitis represents 2 to 4 billion dollars annually in lost revenues to producers (Politis et al. 1995). Dairy cows prior to parturition and slightly thereafter have been shown to have reduced neutrophil functionality, particularly if there was retained fetal membranes (Gilbert et al., 1993). It is therefore critical to attempt to maximize neutrophil functionality of the dairy cow during this time of her life and during other times that she may be experiencing stress.

There has been a great deal of interest lately on the supplementation of antioxidant vitamins such as β-carotene and vitamin E to bolster the immune response of dairy cattle (Batra et al., 1992; Michal et al., 1994). The effect of

antioxidants such as β-carotene and vitamin E seems to be one of maintaining the phagocytic activity of the neutrophils around calving as without supplementation there is a drop in the ability of the neutrophils to ingest and kill microorganisms (Tjoelker et al., 1990).

### 1.3.2.1. INTRACELLULAR INTERACTION BETWEEN NEUTROPHILS AND ASCORBIC ACID

There is considerable research on the relationship between ascorbic acid and neutrophils. Neutrophils which are deficient in ascorbic acid have been shown to have reduced chemotactic ability. Injury and subsequent pathogen invasion may increase circulating levels of neutrophils but the increase number of cells does not result in increased numbers of the phagocytes at the point of invasion because the cells are less able to egress out of the capillaries to the injured site.

Neutrophils that are deficient in vitamin C will produce excess free radicals and will under produce the major antimicrobial agent, hypochlorous acid (Anderson et al., 1990; Marquez et al. 1990). Supplementation with ascorbic acid has been shown to protect neutrophils against free radical damage and enhance function (Bendich et al. 1986). Boxer et al. (1976) showed that the diminished bactericidal and chemotactic abilities of neutrophils from newborns suffering from Chediak-Higashi syndrome could be reversed with the supplementation of vitamin C. The proposed mechanism is that neutrophils from children suffering from this condition

show high levels of cyclic AMP which suppresses neutrophil motility and degranulation. Ascorbic acid reduces cellular cyclic AMP (Boxer et al., 1976).

Ascorbic acid has also been shown to reverse lymphocyte proliferation depression caused by hypochlorous acid. Hypochlorous acid is the major antimicrobial agent possessed by the neutrophil for intra-cellular killing but when it enters the inter-cellular space it reduces the lymphocyte proliferative response. This effect can be reversed by increasing the ascorbic acid concentrations surrounding the lymphocytes (Anderson et al. 1990).

### 1.3.2.2 GLUCOCORTICOIDS AND NEUTROPHIL FUNCTION

Stress has often been associated with the onset of disease. An important response to stress is the release of adrenocorticotropic hormone which in turn causes the adrenal cortex to elaborate corticosteroids (Scott, 1981). Corticosteroid levels are often used as a measure of determining stress in cattle (Gwazdauskas et al. 1980).

It is critical to understand the chain of events which occur when neutrophils are pressed into action if one is to fully appreciate the effect that glucocorticoids may have on the immune response. The steps are: 1) diapedesis, adherence and exit from the vascular epithelium; 2) directed migration along a chemotactic gradient and random migration; 3) capture and engulfment of microorganisms which are

usually opsonized; 4) phagosome and lysosomes fuse together and the lysosomes degranulate into the phagosome; 5) oxidative burst producing hydrogen peroxide, superoxide anion and hydroxyl radicals and singlet oxygen; 6) hydrogen peroxide reacts with halide ions and myeloperoxidase to produce the most potent antimicrobial agent hypochlorous acid and 7) antibody dependent cell mediated cytotoxicity, this allows neutrophils to destroy host cells which are virally infected (Roitt et al. 1986). These processes are critical to the optimal function of neutrophils following exposure to a pathogen and therefore it can be surmised that anything interfering with this cascade of events would lead to reduced neutrophil functionality.

Glucocorticoids, particularly the synthetic forms such as dexamethazone are known for their anti-inflammatory capabilities. In the past it was assumed that glucocorticoids stabilized the lysosomal membranes of neutrophils and hence stopped the release of the reactive oxygen molecules which can cause inflammation (Persulin and Ku, 1974). It is now thought that glucocorticoids mediate inflammation by inhibiting neutrophil cellular metabolism and hence the production of the antimicrobial compounds (Stevenson, 1977).

Glucocorticoids have several negative affects on the overall effectiveness of neutrophil function. In man, glucocorticoids have been shown to decrease the ability of neutrophils to adhere to vascular endothelial cells and therefore the cells are not as likely to egress out of the capillaries to the site of the injury (Clark et al.,

1979). In cattle the administration of ACTH causes transient neutrophilia but the increased number of neutrophils influxing from the bone marrow does not lead to an increased number of neutrophils at the site of the injury (Roth et al., 1982). Gwazdauskas and co-workers (1980) showed that sequential injections of ACTH caused a pronounced neutrophilia in dairy cows.

Roth and Kaeberle (1985), in an elaborate set of studies, examined the influence of a potent synthetic glucocorticoid, dexamethasone, on various neutrophil functions. Neutrophils from cattle injected with dexamethasone and 20 mg/kg body weight ascorbic acid showed significant improvement in oxidative metabolism as measured by the Nitroblue tetrazolium reduction assay as compared to cattle injected with dexamethasone only. The ascorbic acid treated cattle also produced neutrophils that had improved antibody-dependent cell-mediated cytotoxicity, and although not significant, the neutrophils from the ascorbic acid treated group tended to have an increased ability to ingest *Staphylococcus aureus* (Roth and Kaeberle, 1985). These authors suggested that ascorbic acid treatment could be useful in preventing the negative impact that stress or synthetic glucocorticoids can have on the immune system of cattle. Unfortunately the injection of ascorbic acid can often produce severe lesions at the site of injection and is therefore not a practical method of ascorbic acid administration to cattle. A method to deliver ascorbic acid to stressed or glucocorticoid treated animals could prove to be very beneficial to the livestock industry.

## 1.3.3 ASCORBIC ACID AND CORTISOL INTERACTIONS

Depletion of ascorbic acid in steroidogenic tissues occurs following the endogenous release of pituitary trophic hormones such as ACTH or luteinizing hormone (Carballeira et al. 1974). In scorbutic guinea pigs Done et al. (1953) demonstrated that circulating corticosteroid levels were increased ten times. When the animals were treated with ascorbic acid the corticosteroid level returned to normal. It was hypothesized that ascorbic acid inhibited the synthesis of corticosteroids and that the depletion of ascorbic acid in the adrenal glands facilitated steroidogenic activity (Kitabchi, 1967).

Stress causes the release of ACTH from the pituitary which initiates the synthesis of corticosteroids by binding to membrane receptor sites on the adrenal cortex and elevating cellular levels of first, adenylate cyclase and secondly c-Amp (Grahame-Smith et al., 1967; Lefkowitz et al, 1970). Ascorbic acid will reduce the level of ACTH mediated adenylate cyclase levels in the cells of the adrenal cortex (Doulas et al. 1987). Finn and Johns (1980) reported that there was an inverse relationship between steroidogenesis and ascorbic acid content of in vitro bovine adrenal cortex cells after stimulation by ACTH.

A part from its role in reducing intracellular levels of adenylate cyclase, ascorbic acid can reportedly regulate the synthesis of corticosteroids by inhibiting the enzymes C-21 and 11-beta hydroxylases of the adrenal steroidogenic pathway (Kitabchi, 1967). The enzymatic activity required to convert cholesterol to

pregnenolone can also be inhibited by ascorbic acid (Carballeira et al., 1974).

The role of ascorbic acid as a regulator of corticosteroid production was demonstrated in vivo in heat stressed cockerels. Pardue et al. (1985) reported that stressed cockerels supplemented with ascorbic acid had less than half the circulating levels of corticosteroids of the unsupplemented birds. The suggestion was then made that ascorbic acid supplementation could reduce the levels and hence the deleterious effects of corticosteroids in poultry (Pardue et al., 1985).

Ascorbic acid may prove to have similar effects in other species and thus be a novel approach to stress management in domestic animals.

## 1.4. ASCORBIC ACID AND MEAT QUALITY

The retail display of fresh meat is a major point of spoilage for the meat industry and it is reportedly as high as 55% of the total loss of the entire industry (Greer et al. 1994). Consumer acceptance of fresh retail beef is based primarily on the perception that bright cherry red meat is fresh and wholesome. The colour changes of fresh beef originate from the oxidation of oxymyoglobin to metmyoglobin but this process occurs before the meat has reached the point of bacterial spoilage (Nesom-Fleet et al., 1993). Antioxidants which could delay the accumulation of metmyoglobin could greatly increase the consumer appeal of fresh beef and drastically reduce the mark downs and discards which occur because of meat discolouration.

Ascorbic acid and alpha-tocopherol are two naturally occurring antioxidants which have been used endogenously or exogenously to prevent the oxidation of oxymyoglobin in fresh beef.

Shaeffer et al. (1995), reported that feeding beef steers high levels of alpha-tocopherol and infusing the animals with sodium ascorbate immediately before slaughter increased the stability of oxymyoglobin and lipids of muscle tissue. In vitro oxymyoglobin liposome models also demonstrated that the two antioxidant vitamins work synergistically and provided increased antioxidant protection over either vitamin alone when added to the liposome medium (Yin et al., 1993).

There can be complications with using ascorbic acid an antioxidant in meat. Ascorbic acid can act as a prooxidant in lipids at low concentrations and an antioxidant in meat at high concentrations, 140 μM. It has been proposed that ascorbic acid exerts an influence over the oxidation of oxymyoglobin and lipids indirectly. Vitamin C will reductively regenerate the alpha-tocopherol molecule from its free radical form to the biologically active form. It is the alpha-tocopherol molecule which prevents the accumulation of metmyoglobin and the oxidation of lipids.

The infusion of ascorbic acid into feedlot animals prior to slaughter is probably not logistically feasible on a large scale and therefore a dietary ascorbic acid supplement would be beneficial. The inclusion of a rumen stable ascorbic acid form would be a very simple process from a management perspective.

## 1.5 ASCORBIC ACID AND RUMINANTS

Ruminants endogenously produce ascorbic acid and therefore it has generally been assumed that they do not require a supplemental source of vitamin C. Unlike monogastrics, however, the rumen atmosphere destroys essentially all ingested ascorbic acid and therefore the animals are entirely dependent on endogenous production.

In early research trials ascorbic acid sank to very low levels within 2 hours in cows which had 150 grams of ascorbic acid placed directly into their rumens via a fistula (Knight et al. 1941). The in vitro results obtained during this trial also showed a rapid loss of ascorbic acid but it took 4 hours for the complete destruction (Knight et al. 1941). This discrepancy could possibly be explained by the fact that the large quantities of ascorbic acid used in the in vitro work could have lowered the pH of the rumen fluid and thus preserved the vitamin C. Further in vitro investigations of ascorbic acid stability in rumen fluid demonstrated that when starch was added to the fluid the fermentation process caused the pH of the fluid to drop and the break down of ascorbic acid to be slowed (Erb et al., 1947).

### 1.5.1. ASCORBIC ACID IN DAIRY COWS AND MATURE CATTLE

Despite the fact that ruminants can synthesize ascorbic acid there has been documented cases of scurvy in both cows and calves (Cole et al., 1944). Scurvy in cattle causes weight losses, general unthriftiness and dermatosis (Duncan, 1944). Hidiroglou et al. (1977) established a link between low ascorbic acid plasma levels and the stress of exposure to cold in Hereford steers.

Modern day dairy cows have been genetically selected to have very high milk production rates. Concurrently they have been selected for high gluconeogenic synthesizing capability. However, it has never been established whether or not the increased glucose production is used in the mammary gland for the production of lactose or if increased availability of glucose has altered the endogenous production of ascorbic acid. The reported ascorbic acid plasma levels for cows ranges from 1.6 mg/L to 10 mg/L (Itze, 1984).

### 1.5.2. ASCORBIC ACID IN CALVES

Young calves do not synthesize ascorbic acid in any appreciable quantities until they reach the age of 2-3 weeks (Lundquist and Phillips, 1942). The inability of calves to synthesize ascorbic acid explains the results of Cummings and Brunner (1989) that showed calves that were not fed colostrum or vitamin C had greater clinical scores for diarrhea and lower IgG concentrations at 14 days of age than

calves receiving supplemental ascorbic acid.

## 1.6. TRACE MINERAL ASCORBIC ACID INTERACTIONS

Studies regarding the interaction between ascorbic acid and trace minerals are normally carried out with animals with normal ascorbic acid status being given supra nutritional doses of the vitamin. Ascorbic acid influences the absorption and utilization of copper and iron via its role as an antioxidant. Ascorbic acid can reduce ferric iron to ferrous iron which has greater bioavailability to animals. The reverse is true of copper where the reductive power of ascorbic acid renders copper less available (Solomons and Viteri, 1982). In trout, ascorbic acid has been shown to reduce copper toxicities caused by high levels of water borne copper (Hilton, 1984).

Zinc and ascorbic acid interactions are inconclusive as contradictory results regarding absorption have been reported (Solomons and Viteri, 1982).

## 1.7. ASCORBIC ACID PHARMOKINETICS IN RUMINANTS

Research pertaining to the pharmokinetics of ascorbic acid in ruminants is extremely scant. This void in information is unfortunate particularly now when

ascorbic acid is being infused into beef cattle as a method to prolong meat colour on retail display (Shaeffer et al., 1995). Without the basic research on the plasma clearance rates of infused ascorbic acid it is very difficult to predict when cattle should be treated prior to slaughter or stress.

Kolb and colleagues (1992) intravenously infused ascorbic acid into seven healthy cows. The plasma ascorbic acid levels of the cows dropped from 60.4 mg/L at 5 minutes to 18.3 mg/L 120 minutes post infusion. At the last collection made the ascorbic acid plasma levels were still twice the level of baseline. The short duration of this trial does not provide enough information to accurately predict the plasma ascorbic acid clearance rates at lower levels or at longer times post infusion.

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# CHAPTER 2

## Effect of abomasal ascorbic acid infusion on plasma cortisol concentration in lactating dairy cows[1]

### 2.1. INTRODUCTION

Modern day high producing dairy cows face an array of immune compromising stressors. Changing diets, new herd mates and parturition may predispose the cow to increased levels of circulating glucocorticoids, known suppressors of the immune response (Roth and Kaeberle 1982). Glucocorticoids, specifically cortisol compromise many facets of the animal's defense mechanisms. Administration of exogenous glucocorticoids or adrenocorticotropic hormone (ACTH) causes neutrophilia in cattle. The increased number of neutrophils which influx from bone marrow does not lead to enhanced systemic protection.

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[1] A version of this chapter has been submitted for publication. MacLeod, Fedio, Kennelly & Ozimek 1995. *Can. J. Anim Sc.*

The total neutrophil count is increased but the ability of the cells to migrate into the damaged tissue is reduced. Glucocorticoids reduce the ability of the neutrophils to adhere to vascular membranes and thereby reduce the egress of neutrophils from blood into the damaged tissue (Carlson and Kaneko, 1976).

Humoral immune response may also be negatively influenced by glucocorticoids but the cause and effect relationship varies with glucocorticoid type, duration of the elevation, and the nature of the antigen (Roth and Kaeberle, 1982).

Acute stressors activate the hypothalamic-pituitary-adrenal axis resulting in the elaboration of corticotropin releasing hormone (CRH) CRH stimulates the anterior pituitary to release ACTH which in turn causes the adrenal cortex to increase circulating plasma levels of glucocorticoids (Scott, 1981).

Ascorbic acid is thought to have a role in the production and or regulation of corticosteroid production in the bovine adrenal gland (Finn and Johns, 1980). In addition, stress and the associated rise in corticosteroids may reduce the circulating plasma levels of ascorbic acid (Thaxton et al., 1984).

This study was conducted to examine the effect of abomasal infusion of ascorbic acid on the circulating levels of plasma cortisol in lactating dairy cows.

## 2.2. MATERIALS & METHODS

### 2.2.1. ANIMALS

Four rumen fistulated Holstein dairy cows in approximately the same stage of mid lactation were used. The test periods were 48h long and were

followed by 3d of no treatments to allow cortisol and ascorbic acid plasma levels to return to pretrial levels.

The animals used in this experiment were cared for under guidelines comparable to those stated in the guide to the care and use of experimental animals provided by the Canadian Council on Animal Care.

## 2.2.2. TREATMENT/SAMPLE COLLECTION

To approximate the cortisol levels that the cows would experience during stress the animals were injected with 100 I.U. of ACTH every 12h. Plasma samples were collected 1.5h post injection for plasma ascorbic acid, dehydroascorbic acid (DHA) and cortisol determinations. Cows were infused with ascorbic acid at 0, 8, 16 or 24g $d^{-1}$ per animal into the abomasum via rumen fistulas. Blood samples were collected via jugular catheters into heparinzed vacutainers and centrifuged within 1/2h.

## 2.2.3 CORTISOL & ASCORBIC ACID ANALYSES

The cortisol determinations were carried out as outlined by Coat-A-Count Diagnostic Products Corporation, Los Angeles, CA. The ascorbic acid levels were assessed in plasma using a modified version of the procedure of Nagy and Degrell (1989). The column used was a reverse phase Spherisorb OD52, 4x250 mm, 5µm column (Pharmacia Uppsala, Sweden). The pump was a Shimadzu LC-G1 set at

a flow rate of .5 ml $m^{-1}$. Samples were loaded via a syringe loading sample injector with a 20 µl loop (Rheodyne Corporation Model 7125, Cotali, California, U.S.A.). An in line filter (Scientific Systems, Inc. State College Pennsylvania, U.S.A.) was connected between the sample injector and the column. The detector was an LC-4B Amperometric detector (Bioanalytical Systems Inc., West Lafayette, Indiana, U.S.A.) set at .3 V. The running buffer was degassed under vacuum immediately after it was made and subsequently sparged daily with helium. The elimination of gas bubbles from the running buffer is critical as any gas bubbles that reach the detector will cause the baseline to become very unstable. The running time was a minimum of 30 min to avoid extraneous peaks that emerged about minute 23.

### 2.2.4. DEHYDROASCORBIC ACID ANALYSIS

Dehydroascorbic acid (DHA) was determined indirectly by reduction. Samples were reduced with 2,3-dimercapto-1-propanol to convert the DHA to ascorbic acid. The DHA was then calculated by taking the difference between the two measurements.

### 2.2.5. STATISTICAL ANALYSIS

This study was designed as a 4 x 4 Latin square. Statistical analyses were performed using General Linear Models procedure of the SAS Institute, Inc. (1985). All results were reported with least square means $\pm$ SEM.

## 2.3. RESULTS & DISCUSSION

In this study cortisol levels 1.5 h post injection of 100 I.U. of ACTH intramuscularly mimicked results of cows undergoing physiological stress (Gwazdauskas et al. 1980). Despite evidence in other species that ascorbic acid could moderate corticosteroid levels in stressed animals there was no significant effect of the treatments in this trial ($P>0.05$)(Table 2.1).

The daily infusion of 8,16 or 24 g of ascorbic acid into the animals did not produce a significant increase ($P>0.05$) in plasma ascorbic acid levels (Table 2.1). There is, however, a trend which suggests that lactating dairy cows can absorb ascorbic acid from the alimentary tract and are not completely dependent on endogenous production (Table 2.1).

Dehydroascorbic acid plasma levels were not significantly elevated by ascorbic acid infusion ($P>0.05$) (Table 2.1). With respect to the absorption of ascorbic acid there appears to be large animal variability.This may be explained in part by the method of absorption. Humans and other species which are dependant on a dietary supply of ascorbic acid absorb the vitamin actively in the duodenum whereas non scurvy-prone animals are thought to absorb the vitamin by passive diffusion in the ileum (Horning et al., 1984).

## 2.4. CONCLUSIONS

The effect of abomasal ascorbic acid infusion on plasma cortisol

concentrations in lactating dairy cows was investigated. Four rumen fistulated lactating Holstein cows were used in a 4X4 latin square design to assess if abomasal infusion of ascorbic acid could influence plasma cortisol. Plasma cortisol and ascorbic acid were not altered significantly ($P>0.05$) by the infusion of ascorbic acid into the abomasum of adrenocorticotropic hormone treated dairy cows. There was a trend indicating that ascorbic acid can be absorbed by mature dairy cattle post rumen.

**Table 2.1. Effect of abomasal infusion of ascorbic acid on plasma cortisol levels in lactating dairy cows treated with adrenocorticotropic hormone[Z]**

| | Ascorbic acid delivery $g^{-1}head^{-1}day^{-1}$ | | | | P-values [Y] |
|---|---|---|---|---|---|
| Concentrations | Control[X] | 8 | 16 | 24 | |
| n | 4 | 4 | 4 | 4 | |
| **Plasma Ascorbic acid** ($mgL^{-1}$) | 2.92±0.34 | 3.40±0.34 | 3.42±0.34 | 3.91±0.44 | NS |
| **Plasma Dehydro-ascorbic acid** ($mgL^{-1}$) | 1.82±0.11 | 1.85±0.11 | 2.34±0.14 | 2.24±0.11 | NS |
| **Cortisol** ($nmol\ dL^{-1}$) | 20.8±1.30 | 20.2±1.30 | 24.6±1.30 | 22.5±1.60 | NS |

Z Least-square means + SEM

Y NS = No significant difference P>0.05

X Control cows infused with distilled water

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# CHAPTER 3

## Pharmokinetics of ascorbic acid and ascorbyl-2-polyphosphate in the rumen fluid of dairy cows[1]

### 3.1. INTRODUCTION

It is usually assumed that ruminants do not require ascorbic acid from exogenous sources because of their innate ability to produce it. However, it has been postulated that ruminants experiencing stress might be predisposed to a subclinical deficiency of this compound (Hidiroglou et al., 1977). In dairy cattle, especially in early lactation, there is a large demand for glucose, the precursor molecule for ascorbic acid into the mammary gland for the production of lactose. It is not known whether or not the indogenous glucose production of the

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1 A version of this chapter has been submitted for publication. MacLeod, Zhang, Kennelly & Ozimek. *Milchwissenschaft 1996*.

cow is sufficient to meet the metabolic requirements for both ascorbic acid and lactose production (Itze, 1984).

Domestic animals that are experiencing stress often show reduced ascorbic acid levels (Pardue et al., 1985). The reduced levels of circulating ascorbic acid is correlated to increased levels of plasma corticosteroids and a subsequent reduction in the efficiency of the immune response (Nockels, 1988). In cattle that have been injected with synthetic glucocorticoids the negative effect on the neutrophils of the dairy cows could partially be alleviated by the injection of ascorbic acid (Roth and Kaeberle, 1985). Unfortunately, injecting ascorbic acid can often lead to inflammation at the site of injection in cattle and horses (Loscher et al., 1984). It would therefore be useful to examine a method of administering ascorbic acid via oral supplementation. Ascorbic acid in its pure crystalline state is extremely unstable under conditions which exist in the rumen (Knight et al., 1941). It was our intention in this study to compare the stability of crystalline ascorbic acid and a stabilized form of the vitamin, ascorbyl-2-polyphosphate, in rumen fluid in vitro. From this study we will be able to assess whether or not the supplementation of ascorbyl-2-polyphosphate to ruminants is a reliable method of supplying ascorbic acid.

## 3.2. MATERIALS AND METHODS

### 3.2.1. ANIMALS

Rumen fluid from four Holstein dry cows was collected. The animals had

previously been fitted with rumen fistulas. After collection rumen fluid was placed on ice and transported to the lab and subsequently filtered through cheese cloth to remove any large particles and measured in 30 mL aliquots into glass tubes in preparation for the incubations.

### 3.2.2. ASCORBIC ACID AND ASCORBYL-2-POLYPHOSPATE ADDITION

The rumen fluid from each of the 4 cows was used for both the incubations of ascorbic acid and the incubations of the ascorbyl-2-polyphosphate. In total 16 tubes per cow were used. To seven 30 mL tubes from each animal, 200 mg/L of crystalline ascorbic acid was added. To a further seven tubes of rumen fluid, 200 mg/L of ascorbic acid originating from ascorbyl-2-polyphosphate was added. Two tubes were used as the baseline control to determine the level of ascorbic acid in the fluid prior to the additions. One tube per cow per treatment was then analysed for ascorbic acid content according to the following sampling scheme, 0 hour, immediately post ascorbic acid or ascorbyl-2-polyphosphate addition, 1 hr, 2 hr, 4 hr, 6 hr, 12 hr and 24 hr. The samples were incubated at 39°C in an anaerobic environment.

### 3.2.3. ANALYTICAL METHODS

#### 3.2.3.1. ASCORBIC ACID ANALYSES

After incubation the ascorbic acid levels were assessed on samples that had

been diluted 1:100 using Clelands reagent (6% meta-phosphoric acid and .2% dl-dithiotheitol). A modified version of the procedure of Nagy and Degrell (1989) was used. A reverse phase Spherisorb ODS2 C18 column (4X250 mm I.D., 5 µm particles ) was used with a guard cartridge (ODS 4X10, 5 µm), Pharmacia Uppsala, Sweden.The pump was a Shimadzu LC-G1 set at a flow rate of .6 ml $min^{-1}$. Samples were loaded via a syringe loading sample injector with a 20 µl loop (Rheodyne Corporation Model 7125, Cotati, California, U.S.A.). An on-line filter (Scientific Systems, Inc. State College Pennsylvania, U.S.A.) was connected between the sample injector and the pump. The detector was an LC-4B Amperometric detector with a silver/silver chloride reference electrode and a carbon paste working electrode (Bioanalytial Systems Inc., West Lafayette, Indiana, U.S.A.) set at .3 volts. The mobile phase (5% MeOH in 0.1 mM $Na_2HPO_4$, 0.07 mM EDTA $Na_2$, 0.15 mM $CH_3(CH_2)_7OSO_3Na$ adjusted to pH 3.1) was filtered through a 0.45 µm filter, degassed under vacuum immediately after it was made and subsequently sparged daily with helium. The elimination of gas bubbles from the running buffer is critical as any bubbles that reach the detector will cause the baseline to become very unstable. The running time was a minimum of 30 min to avoid extraneous peaks that emerged at about 23 minutes.

### 3.2.3.2. ASCORBIC ACID ANALYSES OF SAMPLES CONTAINING ASCORBYL-2-POLYPHOSPHATE

To analyse the ascorbic acid originating from ascorbyl-2-polyphosphate the phosphate molecules must first be cleaved off. After the rumen fluid was incubated with the ascorbyl-2-polyphosphate 1 mL of the fluid was added to 4 mL of Clelands reagent and stirred for 15 min at room temperature. Part of the solution (1 mL) was then centrifuged in an Eppendorf centrifuge for 20 min. The supernatant, 100 µL was pipetted into ependorph tubes containing 1 mg of acidic phosphatase (Sigma Chemical Co. St. Louis, MO, USA). To this mixture 700 µL of 0.13 M sodium acetate buffer, pH 4.8, containing 0.2% dl-dithiotheitol and 1.1% compressed yeast was added. The sample was then digested for 3 hr at 37°C in a mechanical shaker. After the samples were digested and allowed to cool to room temperature the volume was adjusted to 1 mL by adding 200 µL of .13 sodium acetate buffer, pH 4.8 without the yeast. The sample was then shaken and centrifuged for 10 min in an Eppendorf centrifuge. After centrifugation the sample was diluted 1:1 with 1% meta-phosphoric acid and filtered through a .22 µm filter. The filtrate was then ready for analysis or storage at -70°C. The total dilution for this preparation was 100 times. The samples were then analysed by high performance liquid chromatography and electrochemical detection in an identical procedure to that described in 3.2.3.1. of this thesis.

## 3.3. RESULTS AND DISCUSSION

It has previously been reported that crystalline ascorbic acid has very poor stability in rumen fluid and Knight et al. (1941) determined that ascorbic acid levels essentially fell back to nil after 1-2 hours. Other research has shown that ascorbic acid stability can be dependent on the pH of the fluid, with a lower pH promoting greater stability (Erb et al., 1947).

Figure 3.1. illustrates the stability of crystalline ascorbic acid after various incubation times in rumen fluid. At time 0, immediately post ascorbic acid addition the levels dropped from 200 mg/L to 180 mg/L. After 6 hrs the ascorbic acid levels had essentially declined to trace levels e.g. 6 mg/L. After 12 hrs of incubation no detectable levels of ascorbic acid were present.

Ascorbic acid in the ascorbyl-2-polyphosphate form was found to have reasonably good rumen fluid stability characteristics (Figure 3.2). Immediately after the addition of the ascorbyl-2-polyphosphate the ascorbic acid level was 150 mg/L. This was a loss of 50 mg/L due to processing and the analytical technique (Figure 3.2). The recovery of ascorbic acid from rumen fluid containing ascorbyl-2-polyphosphate was very difficult and therefore the stability results may have been even better with an improved analytical method. After 24 hrs of incubation, 30 mg/L of ascorbic acid still remained in the rumen fluid (Figure 3.2).

Figure 3.3 examines the kinetics of ascorbic acid and ascorbic acid originating from ascorbyl-2-polyphosphate in the rumen fluid. The fluid passage

rates and rumen liquid volume used in the model could be considered average for a dry dairy cow; (Khorasani, 1994). The rumen stability properties of the ascorbyl-2-polyphosphate are shown to be clearly enhanced over the stability of crystalline ascorbic acid (Figure 3.3).

The reaction rate constant of ascorbic acid in rumen fluid is 28.87 mg/L/hr (Figure 3.4). The reaction follows zero order kinetics and as such is completely independent of the initial concentration of ascorbic acid. The half life of ascorbic acid in the rumen can then be calculated by using the integral method for a zero order reaction with the resultant formula $C_A=C_{Ao}- kt$, where $C_A$ is the ascorbic acid concentration, $C_{Ao}$ the initial ascorbic acid concentration, k is the reaction rate constant and t = time. The half life of ascorbic acid in rumen fluid was calculated to be 3.46 h.

Ascorbyl-2-polyphosphate had a reaction rate constant of 0.00096 mg/L/hr (Figure 3.5). The degradation of the ascorbic acid originating from ascorbyl-2-polyphosphate in rumen fluid was to be highly dependent on the initial substrate concentration and followed second order reaction kinetics. The integral method for second order reaction rate constants generated the equation $1/C_A - 1/C_{Ao} = kt$ where $C_A$ = ascorbic acid concentration, $C_o$ = initial concentration, k = reaction rate constant and t = time. The half-life of the ascorbic acid originating from ascorbyl-2-polyphosphate was then calculated to be 5.21 h based on the theoretical concentration at time 0 of 200 mg/L. When the actual analysed concentration of

150 mg/L ascorbic acid at time 0 is used the half-life is 6.94 h and is thought to be a more accurate description of the stability of the compound in rumen fluid.

The conclusion that may be drawn from this data is that ascorbyl-2-polyphosphate has much greater rumen stability than crystalline ascorbic acid and therefore holds promise as a potential oral supplement of vitamin C for ruminants.

## 3.4. CONCLUSIONS

Ascorbic acid crystalline and ascorbic acid originating from ascorbyl-2-polyphosphate were incubated in rumen fluid to determine the stability properties of both compounds. Based on the reaction rate constants and the half-lives of both vitamin forms it can be concluded that ascorbyl-2-polyphosphate is a more rumen fluid stable form of ascorbic acid. Due to the stability, ascorbyl-2-polyphosphate may prove usefull as a souce of orally administered vitamin C.

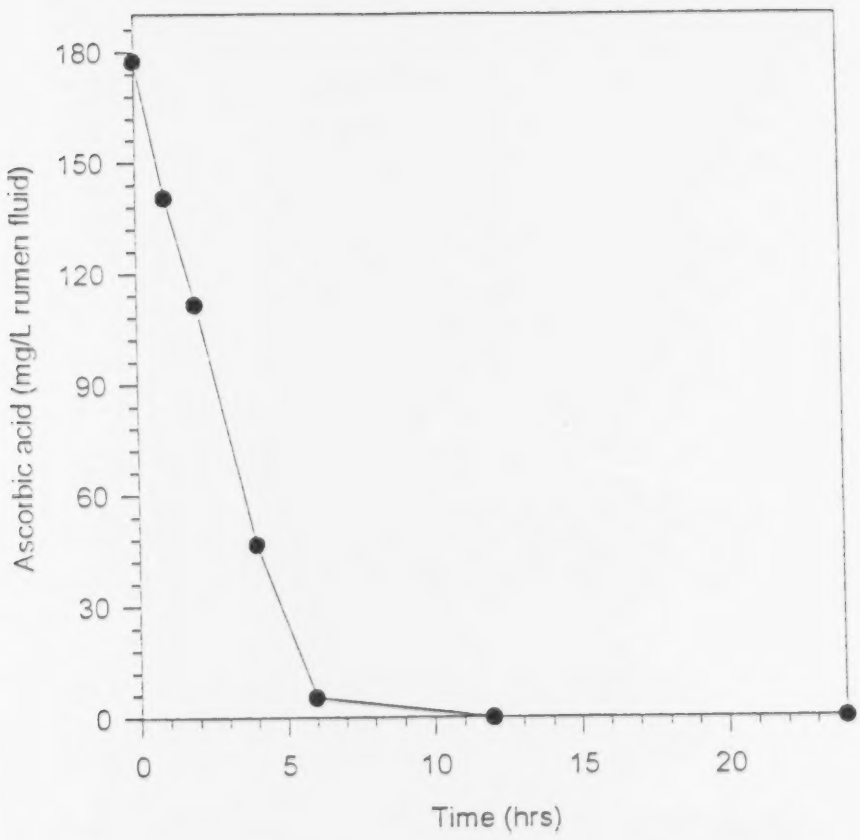


Figure 3.1: The disappearance of ascorbic acid in rumen fluid of 4 cows over 24 hours.

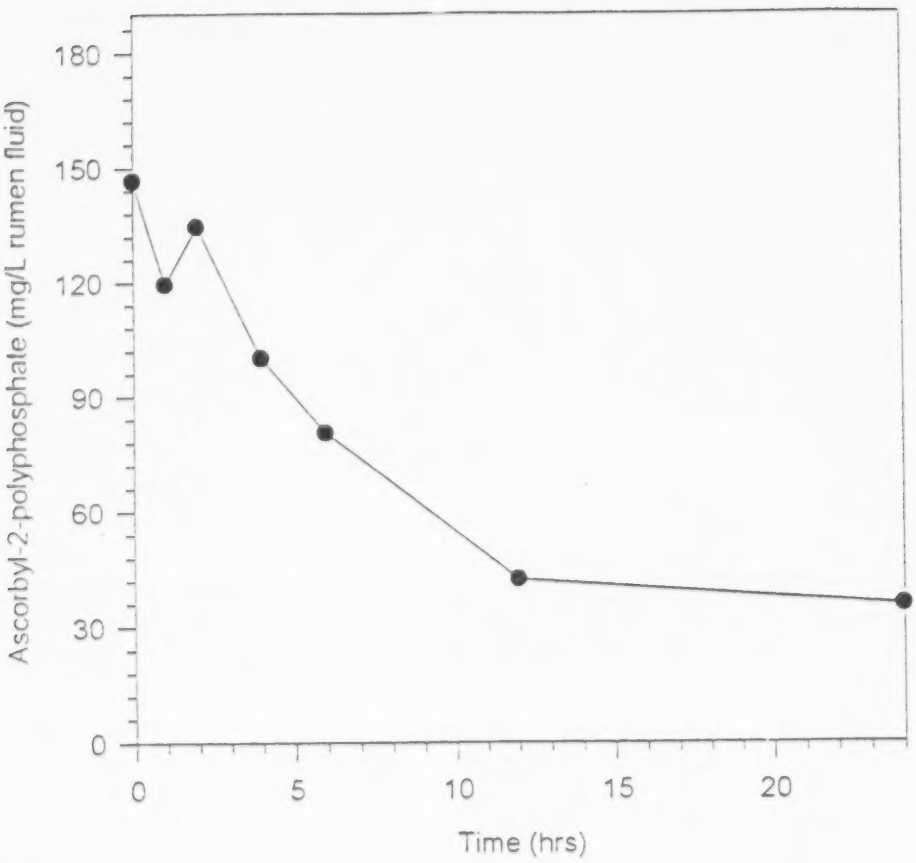


Figure 3.2: The disappearance of ascorbyl-2-polyphosphate in rumen fluid of 4 cows over 24 hours.

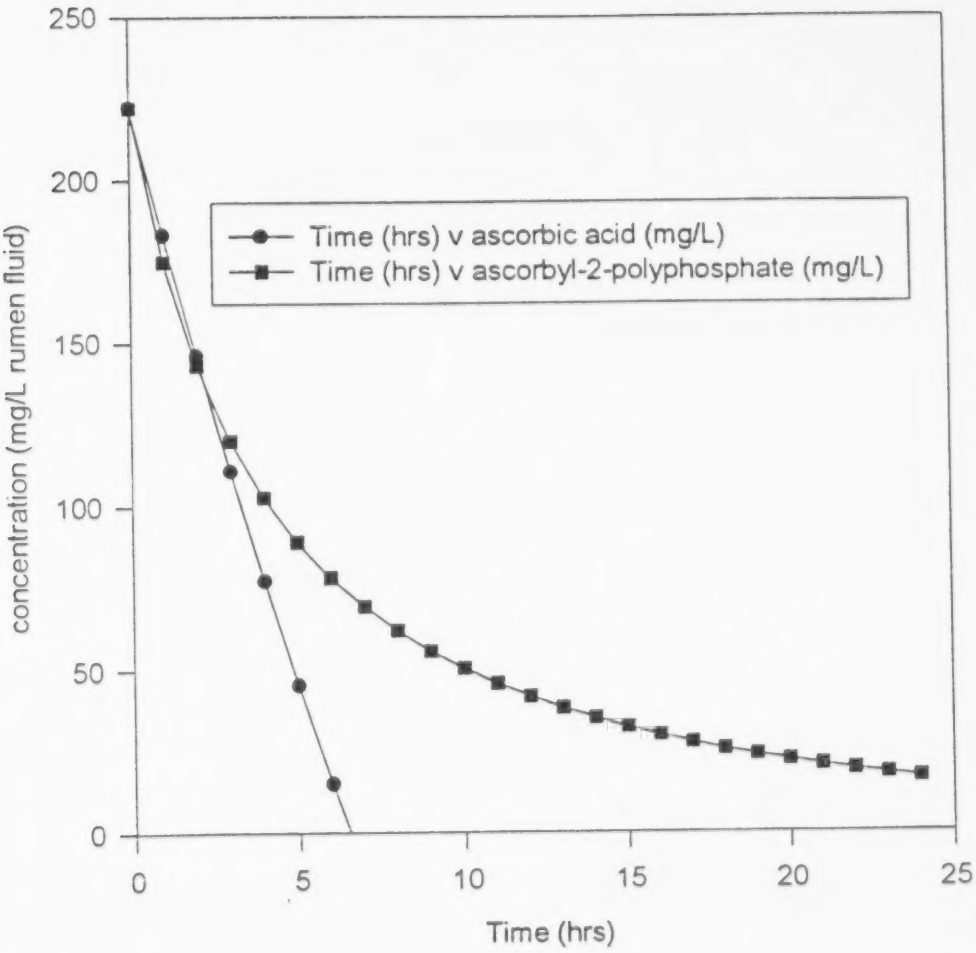


Figure 3.3: Estimated ascorbic acid and ascorbyl-2-polyphosphate concentration in the rumen of cows over 24 hours based on 90L capacity, constant in/out flow rate of 4.5L/hr and 200mg/L rumen fluid substrate concentration

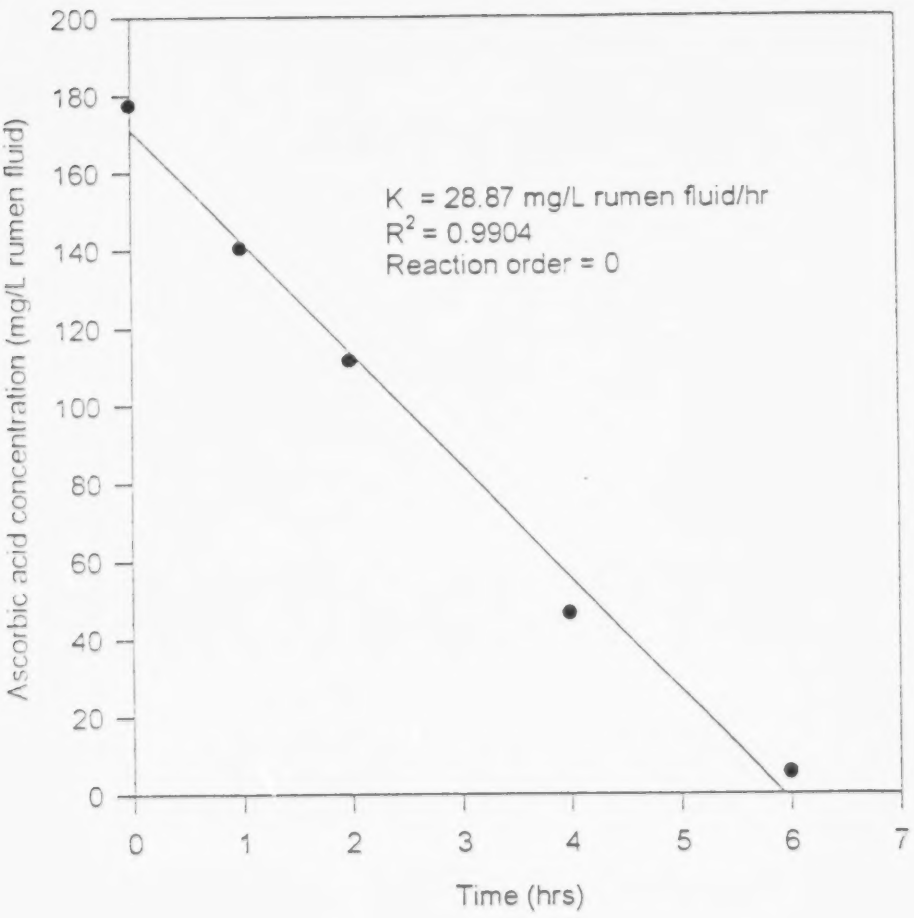


Figure 3.4: The reaction rate constant of ascorbic acid in the rumen fluid of 4 cows

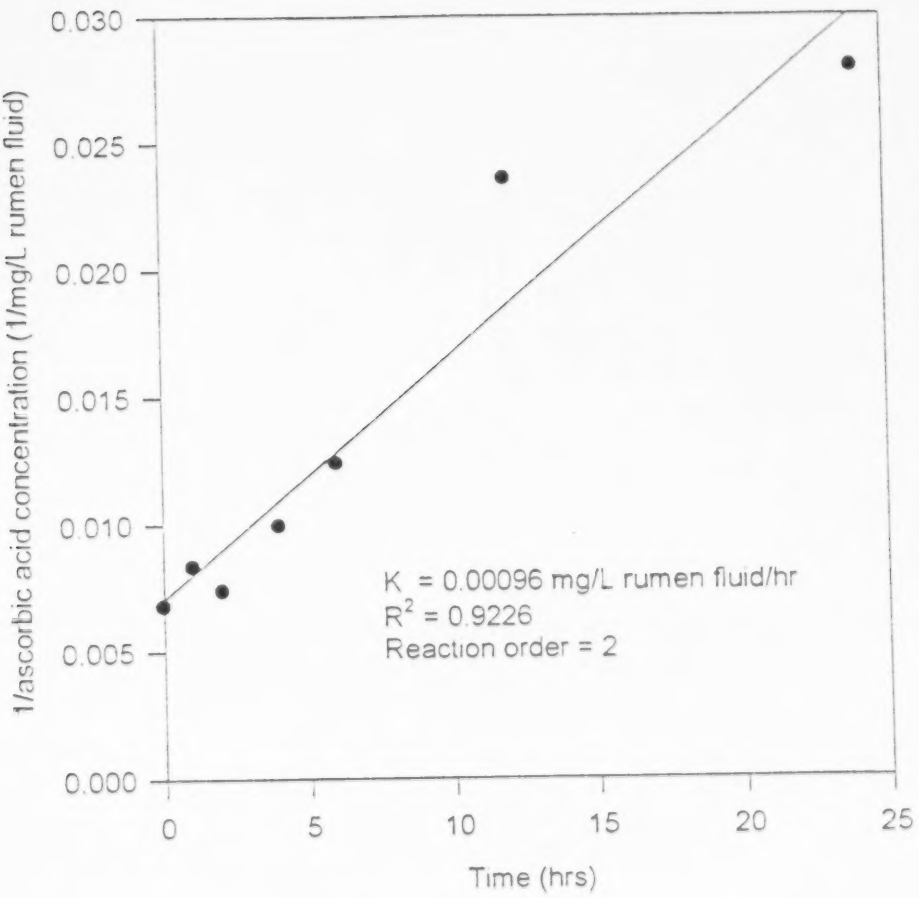


Figure 3.5: The reaction rate constant of ascorbic acid originating from ascorbyl-2-polyphosphate in the rumen fluid of 4 cows

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# CHAPTER 4

## Disappearance rate of ascorbic acid injected into the jugular vein of dairy cows

### 4.1. INTRODUCTION

The injection of ascorbic acid into the jugular vein of cattle is the most practicle and efficacious method to raise the plasma content of the compound (Kolb et al., 1992). Intra-muscular and subcutaneous injections have been tried in other species with variable results and often caused severe inflammation at the injection site (Loscher et al., 1984).

Ascorbic acid has been shown to be beneficial in ameloriating the negative impact of stress on the immune system of many classes of domestic livestock including cattle (Cummins and Brunner, 1989). Cattle subjected to synthetic glucocorticoid injection showed improved neutrophil function if the cattle were

treated with ascorbic acid (Roth and Keberle, 1985).

Recently there has been renewed interest in using ascorbic acid to enhance the quality of beef (Yin et al., 1993). In vitro assessments of vitamins C and E combinations showed a significant improvement in colour retention and a reduction of lipid oxidation in fresh beef (Yin et al., 1993). Ascorbic acid has also been injected into beef animals just prior to slaughter and the resultant increase in meat ascorbic acid has improved the shelf life and quality of the meat (Schaeffer et al., 1995).

This experiment was designed to assess the degradation rates of ascorbic acid after the infusion of sodium ascorbate into the jugular vein of dairy cattle. This data will allow future researchers to predict plasma ascorbic acid levels at any particular time after an intravenous injection. The practicle ramifications are two-fold: 1) in cattle that are going to being experiencing stress, the timing of ascorbic acid supplementation could be calculated and 2) the timing of pre-slaughter injections could be calculated to ensure optimum plasma and meat levels of vitamin.

## 4.2. MATERIALS AND METHODS

### 4.2.1. CATTLE

Four Holstein cows weighing 706 kg $\pm$ 45 and in approximately the same stage of mid lactation were used for this study. Cows were fitted with indwelling jugular catheters to minimize stress during the infusion of ascorbic acid and the

subsequent blood sampling.

### 4.2.2. INFUSION TECHNIQUE

Sodium ascorbate (Hoffmann-LaRoche, Ltd. Miss. Ont.) with an ascorbic acid concentration of 87.7% was disolved in sterile physiological saline at a concentration 300 mg/mL. The cattle were then infused with the solution to deliver 10 mg/kg of body weight. The infusion took approximately 1 min to deliver. Prior to infusion blood samples were collected to establish base line plasma ascorbic acid levels. Blood samples were collected post infusion at .167, .5, .83, 1.5, 2.67, 5.33, 8.0, 12.0, 16.0 and 24 h into 10 mL heparinized vacutainers.

### 4.2.3. ASCORBIC ACID ANALYSES

After the samples were centrifuged for 10 min at 15,000Xg the plasma was harvested and diluted 1:100 in Clelands reagent (6% meta-phosphoric and .2% dl-dithiotheitol) and stored at -70°C until analysis. The time which elapsed between blood collection and sample storage was always less than .5 hr.

Ascorbic acid plasma levels were assessed using a modified version of the high performance liquid chromatographic procedure of Nagy and Degrell (1989) A reverse phase Spherisorb ODS2 C18 column (4X250 mm I.D., 5 µm particles ) was used with a guard cartridge (ODS 4X10, 5 µm) (Pharmacia Uppsala, Sweden).The pump was a Shimadzu LC-G1 set at a flow rate of .6 ml $min^{-1}$.

Samples were loaded via a syringe loading sample injector with a 20 µl loop (Rheodyne Corporation Model 7125, Cotati, California, U.S.A.). An on-line filter (Scientific Systems, Inc. State College Pennsylvania, U.S.A.) was connected between the sample injector and the pump. The detector was an LC-4B Amperometric detector with a silver/silver chloride reference electrode and a carbon paste working electrode (Bioanalytial Systems Inc., West Lafayette, Indiana, U.S.A.) set at .3 volts. The mobile phase (5% MeOH in 0.1 mM $Na_2HPO_4$, 0.07 mM EDTA $Na_2$, 0.15 mM $CH_3(CH_2)_7OSO_3Na$ adjusted to pH 3.1) was filtered through a 0.45 µm filter, degassed under vacuum immediately after it was made and subsequently sparged daily with helium. The elimination of gas bubbles from the running buffer is critical as any bubbles that reach the detector will cause the baseline to become very unstable. The running time was a minimum of 30 min to avoid extraneous peaks that emerged at about 23 minutes.

## 4.3. RESULTS AND DISCUSSION

Figure 4.1 illustrates the rapid decline in plasma ascorbic acid levels which occurs post injection. The majority of the ascorbic acid appears to be degraded or distributed to peripheral tissues within 0.5 hr after the injection. Figure 4.1. also illustrates that ascorbic acid distributes in the body according to a two-compartment model. Ascorbic acid entering the central compartment which consists of plasma and the extracellular fluid of highly perfused organs rapidly equilibrates and then

begins to distribute to less well perfused tissues such as muscle (Loscher et al., 1984).

It is the distribution of ascorbic acid in the terminal compartment that we are primarily interested in as this parameter will have the greatest impact on meat quality. The distribution of ascorbic acid can best be described by the integral method for a second order reaction rate constant where $1/C_A - 1/C_{A_0} = kt$ where $C_A$ = ascorbic acid concentration, $C_{A_0}$ = initial concentration, k = reaction rate constant and t = time. From figure 4.2 the half-life of the terminal compartment can be calculated as was found to be 11.4 hr. The half-life of ascorbic acid in the terminal compartment is similiar to that reported in horses (Loscher et al., 1984).

Based on the data in figures 4.1 and 4.2 it can be calculated that cattle injected with 10 mg/kg body weight will have plasma levels appreciably above the physiological norm for at least 12 hr. Future research will need to be conducted to determine if the time frame in which ascorbic acid is elevated in the animals is of significant enough duration to increase the levels in muscle tissue.

## 4.4. CONCLUSIONS

Four Holstein dairy cows were injected with ascorbic acid at 10 mg/kg body weight. Ascorbic acid distributed itself according to a two compartment open model. The terminal half-life was determined to be 11.4 h.

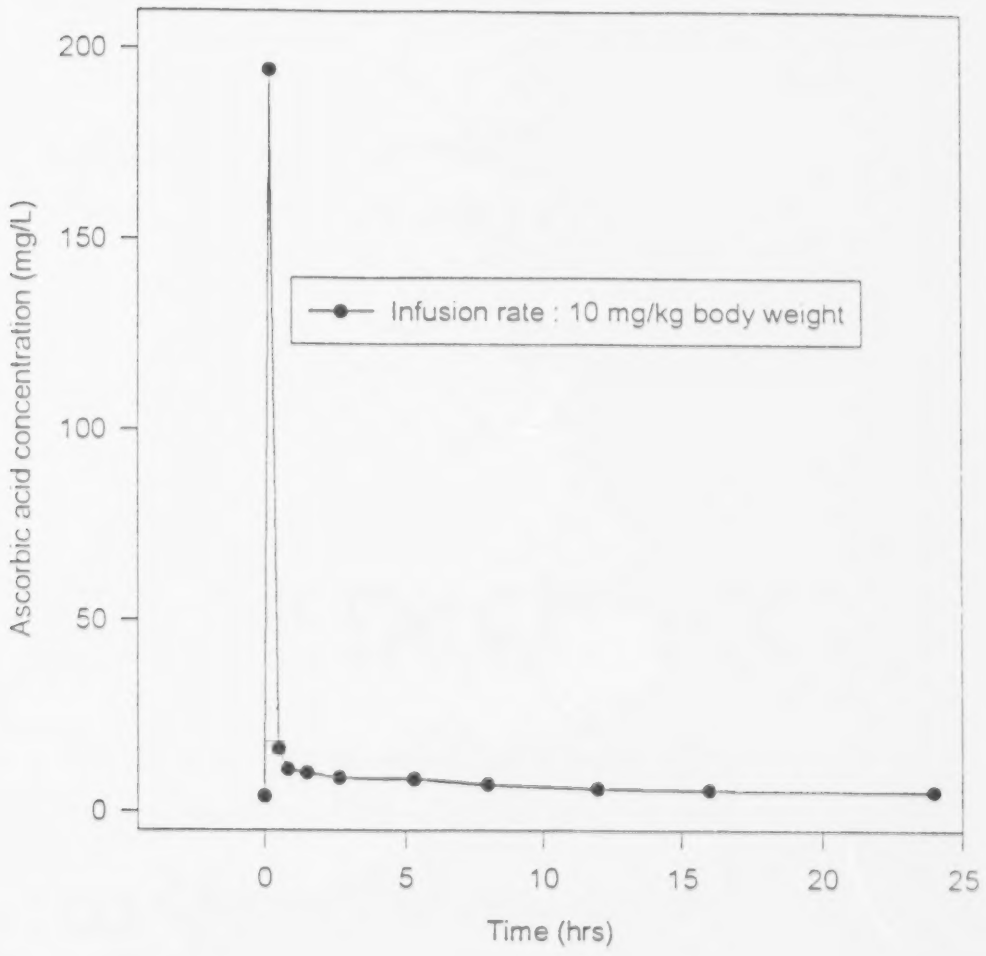


Figure 4.1: The disappearance of sodium ascorbate (mg/L) after infusion into 4 cows

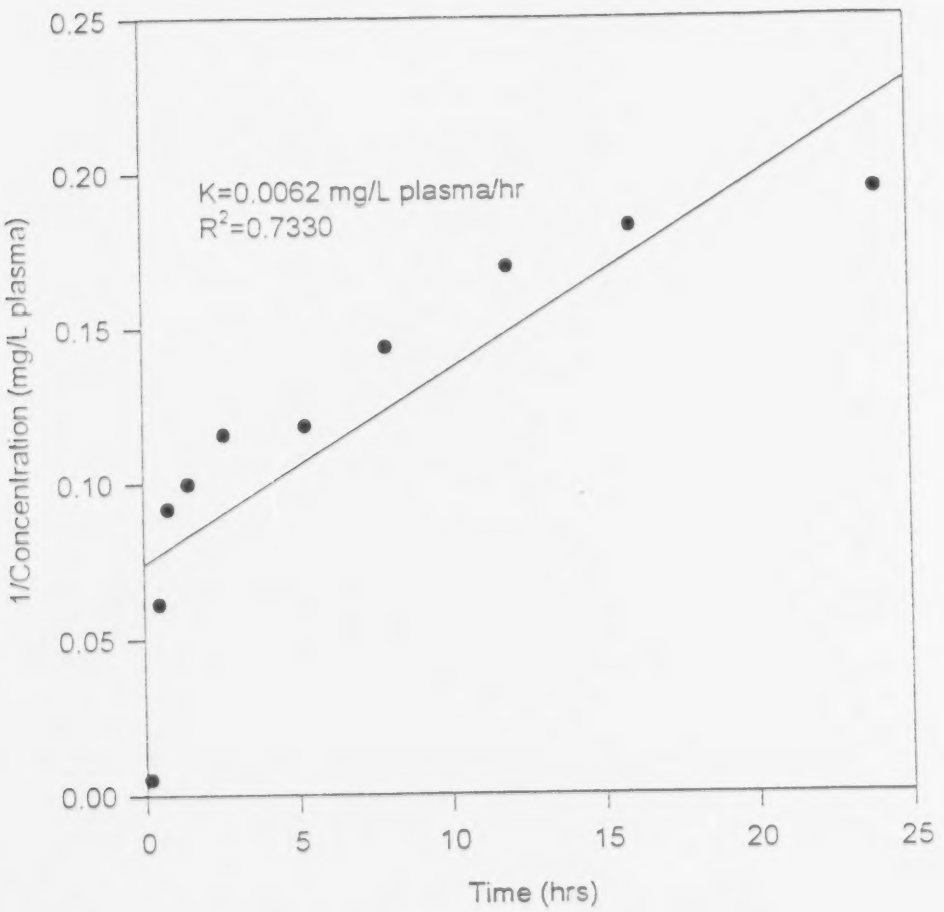


Figure 4.2: The reaction rate constant of ascorbic acid infused into the jugular vein of 4 cows

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# CHAPTER 5

## Separation of purified bovine polymorphonuclear leukocytes with Ficoll-Paque

### 5.1. INTRODUCTION

The isolation of purified viable neutrophils from the whole blood of dairy cows is a critical step towards a better understanding as to how the innate disease fighting mechanisms of the cow might be enhanced. To date, separation procedures for bovine neutrophils were time consuming and required relatively large quantities of blood (Roth and Kaeberle, 1981). One step Hypaque-Ficoll Separation methods that are currently used to

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[1] A version of this chapter has been published. MacLeod, Janowska-Wieczorek, Fedio, Ozimek & Kennelly, *Milchwissenschaft* **50(10)** :547-548 1995.

rapidly separate human polymorphonuclear leukocytes (PMN) are of limited value when assessing bovine blood as the specific gravity of bovine PMN are similar to the specific gravity of the erythrocytes and therefore good separations cannot be obtained (Ferrante and Thong, 1982; Bignold and Ferrante, 1987).

This work describes the use of Ficoll-Paque medium and subsequent centrifugation to obtain consistent purity and high yields of PMN from heparinized bovine blood.

## 5.2. MATERIALS & METHODS

### 5.2.1. BLOOD SAMPLES

Samples of whole blood(5) were collected via an indwelling jugular catheter into heparinized 10 ml glass tubes and mixed well. The donor cow was a Holstein in approximately the 120th day of lactation. All samples were analyzed within 4 hours of collection.

### 5.2.2.PREPARATION OF LEUKOCYTE RICH FRACTION

Whole blood samples (10 ml) were centrifuged at 300Xg for 10 minutes (I.E.C. Clinical Centrifuge, Damon/I.E.C. Needham Mass. USA). The majority of the plasma was then removed leaving approximately 1.2 cm

of the material above the buffy coat layer. The remaining plasma, buffy coat layer and the top 1-2 cm of the erythrocyte layer was then transferred by pasteur pipette (2.3 ml) into a sterile tube and diluted with Iscove's medium (4.5 ml).

The diluted leukocyte rich solution was gently layered onto 5 ml of Ficoll-Paque, specific gravity 1.077g/ml (Pharmacia LKB Biotechnology Inc., Pisctaway, New Jersey, USA). A Beckman model TJ-6 centrifuge and TH4 rotor (Beckman Instruments Inc., San Ramon CA, USA) was used to spin the ficoll density gradient for 30 min at 400 g. Rotor speed was increased gradually as to not disturb the cellular-Ficoll interface. Upon completion of centrifugation, distinct bands of varying cell types emerged. The upper most section of the tube was media followed by a band of lymphocytes and then the Ficoll-Paque. Beneath the ficoll was a pellet of erythrocytes and PMN (Roitt et al. 1986).

### 5.2.3. FINAL PMN PREPARATION

All material except for the erythrocyte and PMN pellet was discarded. The pellet was washed with Iscove's media (5 ml) and centrifuged for 5 min at 300 g in the I.E.C. Clinical centrifuge to obtain the pellet. Washing of the pellet was then repeated to remove all traces of the Ficoll-Paque.

The washing medium is then removed and a lysis solution (10 ml)

consisting of 8.29 g/L $NH_4Cl$, 0.84 g/L $NaHCO_3$ and 0.0372 g/l $Na_2EDTA$ was added to resuspend the pellet and lyse the erythrocytes. Subsequent to a further 300Xg I.E.C. spin and additional 5 ml of the lytic solution can be added to the pellet if there appears to be residual red blood cell contamination. The remaining PMN pellet was then washed in Iscove's medium (3 ml) and I.E.C. centrifuged for 5 min at 300 g. The washed pellet was resuspended in fresh Iscove's medium (1 ml).

### 5.2.4. LEUKOCYTE COUNTS

A Coulter counter (Coulter Electronics Inc., Hialeah Florida, USA) was used to obtain the leukocyte counts in the initial whole blood, the leukocyte rich fraction and the final PMN preparation. Zap-oglobin II (Coulter Electronics Inc., Hialeah Florida, USA) (200 µl) was added to each dilution sample to eliminate the erythrocytes. The samples were first prepared by adding 40 µl of the test material to 20 ml of diluent. Three counts were obtained from every sample.

### 5.2.5. PMN PURITY ASSESSMENT

Differential counts of PMN and monocytes were completed on whole and separated cow's blood. Counts were made using a Leitz (Wetzlar) microscope at 100X. Slides were prepared using cytospin slides stained

with Wright's stain.

## 5.3. RESULTS & DISCUSSION

Blood from a Holstein dairy cow in day 120 of lactation was collected and the distribution of leukocytes in whole and separated blood was assessed (Table 5.1). The percentage of various leukocytes in whole blood were found to fall within normal ranges for a cow of this age and breed (Schalm et al. 1975). The results of the whole blood separation also suggested that the cow was habituated to the blood collection procedures as there was not any apparent neutrophilia (Gwazdauskas et al. 1980).

The separation procedure yielded highly purified PMN (Table 5.1). The total PMN count of the separated blood was 97.6% and one standard deviation was 1.0 (Table 5.1). The main contaminant of the purified PMN were the lymphocytes at 2.4%; there were not any monocytes in any of the samples examined.

The percentage recovery of the total PMN from whole blood was 23.9% (Table 5.2). Segmented or mature neutrophil recovery was 25.2%. Eosinophil and basophil recovery were 20.6 and 17.5% respectively (Table 5.2).

The purity of the PMN fraction makes this separation technique very useful for subsequent immunological assays.

## 5.4. CONCLUSIONS

Studies were conducted to selectively isolate polymorphonuclear leukocytes from bovine blood. Following a short, low speed centrifugation a leukocyte rich fraction was collected. This fraction was diluted 1:1 with Iscove's Modified Dulbecco's Medium prepared and layered onto Ficoll-Pacque and centrifuged. Polymorphonuclear leukocytes and erythrocytes migrated through the density gradient and formed a pellet at the bottom of the tube. Erythrocytes were selectively lysed leaving a highly purified polymorphonuclear leukocyte preparation. The purity and viability of the resultant polymorphonuclear leukocyte fraction makes this technique very useful for subsequent immunological assays.

Table 5.1. Distribution (%) of leucocytes in whole and separated blood of a lactating dairy cow

| Cell Type | Whole Blood (n=5) | Separated Blood (n=5) |
|---|---|---|
| Segmented neutrophils | 29.2 (± 1.2)[z] | 85.6 (± 2.3) |
| Band neutrophils | 1.4 (± 1.5) | 1.8 (± 0.4) |
| Eosinophils | 3.8 (± 1.3) | 7.4 (± 1.4) |
| Basophils | 2.0 (± 1.5) | 2.8 (± 0.9) |
| Total PMN | 36.4 (± 2.7) | 97.6 (± 1.0) |
| Lymphocytes | 44.6 (± 3.3) | 2.4 (± 1.0) |
| Monocytes | 17.0 (± 3.5) | 0 |

z ± 1 standard deviation

Table 5.2. Average percentage recovery of purified polymorphonuclear leucocytes (PMN) from the blood of a lactating cow

| Cell Type | Whole blood PMN count per ml x $10^6$ (n=5) | PMN count per ml x $10^6$ Separated Fraction (n=5) | % Recovery (n=5) |
|---|---|---|---|
| Segmented Neutrophil | 1.876 (± .11)[z] | .47 (± .800) | 25.22 (± 5.04) |
| Band Neutrophils | 0.089 (± .10) | .01 (± .003) | 5.00 (± 6.04) |
| Eosinophils | 0.091 (± .24) | .04 (± .010) | 20.57 (± 10.83) |
| Basophils | 0.101 (± .13) | .016 (± .008) | 17.48 (± 9.52) |
| Total PMN | 2.284 (± .19) | . 540 (± .100) | 23.86 (± 5.48) |

z ± 1 standard deviation

## 5.5. REFERENCES

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# CHAPTER 6

## Ascorbyl-2-polyphosphate as a source of ascorbic acid for dairy cattle[1]

### 6.1. INTRODUCTION

Generally, it has been assumed that endogenously produced ascorbic acid was sufficient to meet the metabolic demands of ruminants. However, recent research on swine and poultry (Yen and Pond, 1981; Pardue et al., 1985) suggests that under specific environmental and physiological conditions the amount of ascorbic acid produced by the animal may in fact be insufficient to meet its requirements. Lactating dairy cattle may be predisposed to subclinical ascorbic acid deficiency because of the demand for the precursor molecules for ascorbic acid production, glucose and galactose into milk. Non-lactating ruminants may also

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1 A version of this chapter has been submitted for publication. MacLeod Zhang, Kennelly & Ozimek. Milchwissenschaft 1996.

have their ascorbic acid status compromised during periods of stress (Hirdiroglou et al., 1977).

The physiological ramifications of subclinical ascorbic acid deficiency are multifactorial. Ascorbic acid is critical if the effectiveness of the immune system is to be optimized (Bendich, 1992; Bendich, 1989). Pathogenic organisms such as bacteria and viruses cause increased free radical production in the infected host and a concomitant decrease in ascorbic acid level and immune function (Beisel, 1982).

Neutrophils are the primary leukocytes which are responsible for the killing of invading pathogens. A deficiency of ascorbic acid can reduce the ability of the cell to migrate to the site of the inflammation, allowing for increased oxidative damage to the neutrophils and reduced production of the major antimicrobial agent, hypochlorous acid. Hypochlorous acid can also exert a negative effect on lymphocyte proliferation. In the presence of adequate levels of ascorbic acid lymphocyte proliferations occur normally (Anderson et al., 1990). Ascorbic acid and other antioxidant vitamins such as vitamin E have been shown to enhance neutrophil function and minimize free radical damage (Politis et al., 1995).

Ascorbic acid may also modulate the immune system via its role in the regulation of hormones associated with stress. Acute stressors activate the hypothalamic-pituitary-adrenal axis resulting in the elaboration of corticotrophin releasing hormone (CRH). CRH stimulates the anterior pituitary to release

adrenocorticotropic hormone which in turn causes the adrenal cortex to increase circulating plasma levels of glucocorticoids (Scott, 1981). Glucocorticoids, specifically cortisol, compromise many facets of the cellular and humoral immune response.

Vitamin C is thought to have a role in the production and regulation of corticosteroid production in the bovine adrenal gland (Finn and Johns, 1980). In addition, stress and the associated rise in corticosteroids may reduce the circulating plasma levels of ascorbic acid (Pardue et al., 1985).

Vitamin C is a known antioxidant and in this capacity can quench free radicals and thereby protect the structural integrity of the cells of the immune system (Bendich et al., 1986). The antioxidant properties of ascorbic acid may also impart improved quality to beef (Yin et al., 1993). Cattle fed high levels of another antioxidant vitamin, alpha-tocopherol, produced steaks that had superior shelf life in terms of colour and lipid oxidation (Arnold et al., 1992). There is a close synergism between ascorbic acid and vitamin E in that vitamin C can reduce the radical of vitamin E back to its active reduced state (Niki et al., 1984). Vitamin C, therefore, may play a two fold role in preventing the oxidation of the meat colour pigment, oxymyoglobin, to the less desirable metmyoglobin. Metmyoglobin is the pigment which causes fresh beef to become brown in colour thus reducing its consumer appeal. Ascorbic acid may enhance the quality of beef directly by acting as antioxidant and indirectly by preserving the antioxidant potential of vitamin E.

Ascorbic acid has been shown to have interactions with trace minerals in species other than ruminants (Hilton, 1984). In particular, there has been considerable research on the interaction between iron and ascorbic acid in swine (Yen and Pond, 1981). Intestinal absorption of nonheme iron is known to increase in the presence of ascorbic acid (Yen and Pond, 1981). Iron is transferred into mucosal cells in the ferrous form and because vitamin C can reduce ferric iron to ferrous iron it may facilitate absorption (Yen, 1984).

In contrast to iron, copper absorption may actually be reduced by ascorbic acid. Vitamin C can reduce the divalent copper ion to the less absorbable monovalent ion (Yen, 1984).

There has been far less research conducted on any interactions that may occur between zinc absorption and ascorbic acid. The studies that have been completed in this area are often contradictory in nature (Solomons and Viteri, 1982).

The present study was conducted to determine if feeding ascorbyl-2-polyphosphate to dairy heifers would alter the plasma, muscle and neutrophil ascorbic acid content. Furthermore, the effect of ascorbic acid on trace mineral plasma levels was examined.

## 6.2. MATERIALS & METHODS

### 6.2.1. ANIMALS

Twelve Holstein dairy heifers weighing 372 $\pm$ 46 kg were used. Six animals

were fed 80 g of ascorbyl-2-polyphosphate (Hoffmann LaRoche Ltd., Mississauga, Ont.) or 20 g ascorbic acid equivalent for 31 days. The animals were individually housed and hand fed for the duration of the trial. The animals used in this experiment were cared for under guidelines comparable to those stated in the guide to the care and use of experimental animals provided by the Canadian Council of Animal Care.

### 6.2.2 BLOOD COLLECTION AND SAMPLE PREPARATION

Blood was collected from all 12 animals prior to the commencement of the trial by jugular venipuncture. Blood was collected in 10 ml heparinized vaccutainers and placed on ice. After centrifugation the plasma was diluted 1:10 using Clelands reagent which consists of 6% meta-phosphoric acid and .2% dl-dithiotheitol ( DTT). The samples were then stored at -70°C and analyzed within two weeks. The same procedure was used 31 days later at the end of the trial period.

### 6.2.3. MUSCLE SAMPLE COLLECTION & PREPARATION

The surgical biopsies were performed on the biceps femoris of each heifer in a manner similiar to the method of Jensen (Jensen, 1989). In brief, a 2 cm incision was made to expose the muscle and a 0.5 g sample was obtained. This sample was divided in half and each segment weighed and placed in 5 ml of 6% metaphosphoric acid and .2% DTT. The samples were homogenized, filtered (.22

μm) and stored at -70°C until analysis. The biopsies were also performed at the end of the trial period at the bicep femoris but on the other hip.

### 6.2.4. NEUTROPHIL SEPARATION

Blood collected from the 12 heifers at the beginning and end of the trial was separated to obtain purified neutrophils by the method described by MacLeod et al., (1995). The neutrophils were counted by hemocytometer and diluted with 2 ml of 6% metaphosphoric acid and .2% DTT and frozen at -70°C. Analyses were completed within 2 weeks of harvest. All samples were analysed in duplicate.

### 6.2.5. ASCORBIC ACID ANALYSES

The ascorbic acid levels were assessed in the plasma, muscle and neutrophils using a modified version of the high performance liquid chromatography procedure of Nagy and Degrell, (1989). A reverse phase Spherisorb ODS2 C18 column (4X250 mm I.D., 5 μm particles ) was used with a guard cartridge (ODS 4X10, 5 μm) (Pharmacia Uppsala, Sweden). The pump was a Shimadzu LC-G1 set at a flow rate of .6 ml $min^{-1}$. Samples were loaded via a syringe loading sample injector with a 20 μl loop (Rheodyne Corporation Model 7125, Cotati, California, U.S.A.). An on-line filter (Scientific Systems, Inc. State College Pennsylvania, U.S.A.) was connected between the sample injector and the pump. The detector was an LC-4B Amperometric detector with a silver/silver chloride reference

electrode and a carbon paste working electrode (Bioanalytial Systems Inc., West Lafayette, Indiana, U.S.A.) set at .3 volts. The mobile phase (5% MeOH in 0.1 mM $Na_2HPO_4$, 0.07 mM EDTA $Na_2$, 0.15 mM $CH_3(CH_2)_7OSO_3Na$ adjusted to pH 3.1) was filtered through a 0.45 µm filter, degassed under vacuum immediately after it was made and subsequently sparged daily with helium. The elimination of gas bubbles from the running buffer is critical as any bubbles that reach the detector will cause the baseline to become very unstable. The running time was a minimum of 30 min to avoid extraneous peaks that emerged at about 23 minutes.

### 6.2.6. PLASMA, IRON, COPPER & ZINC DETERMINATION

Trace mineral determinations in plasma were performed by Atomic Absorption Spectrophotometry (Model 400 Perkin-Elmer Corporation, Norwalk, CT, USA).

### 6.2.7. STATISTICAL DESIGN

Six control animals and six test animals were used in a T-test design (Steele and Torrie, 1980). Differences between means, $P<0.05$, were considered to be significant.

## 6.3. RESULTS & DISCUSSION

When animals are under stress the endogenous production of ascorbic acid

may not be sufficient to meet the requirements of the animal (Pardue et al., 1985). Due to the fact that free ascorbic acid is rapidly destroyed in the rumen there was not any practical method to supplement ruminants with oral vitamin C (Vanich et al., 1945; Knight et al., 1941). Ascorbyl-2-polyphosphate appears to be an ascorbic acid compound which has sufficient rumen stability to increase ascorbic acid plasma levels of dairy heifers (Table 6.1). Prior to the commencement of the trial the plasma ascorbic acid levels of the control and test groups were 3.83 and 3.72 mg/L respectively ($P>0.05$). Following the top dressing of 80 g per head per day of ascorbyl-2-polyphosphate for 31 days the test animals had a significant increase in ascorbate plasma levels over the control animals, 4.56 versus 3.58 mg/L, respectively ($P<0.05$)(Table 6.1). These results indicate that ascorbyl-2-polyphosphate can be used as an ascorbic acid supplement for ruminants. The fact that the plasma levels remained elevated even after 31 days suggests that the animals did not compensate for the exogenous supply of ascorbic acid by reducing their endogenous production of the vitamin.

Biopsies of the biceps femoris of the heifers were performed at the beginning and end of the trial. The reasons biopsies were required were: 1) an additional indication as to the bioavailability of the ascorbyl-2-polyphosphate and 2) to determine the possible use of this product formula to raise meat ascorbic acid levels. Increased meat ascorbic acid may improve the quality of retail beef (Yin et al., 1993). Initially there was no difference between the tissue ascorbic acid levels

of the test and control animals, 7.80 versus 8.10 mg/Kg wet weight (P>0.05)(Table 6.2). After the treatment there was a trend for the animals receiving ascorbic acid to have increased levels of muscle ascorbic acid. The tissue levels were 10.11 and 8.32 mg/Kg ascorbic acid for the test and control animals (P=.08)(Table 6.2). The results of the plasma and muscle ascorbic acid analyses together form a compelling argument that ascorbyl-2-polyphosphate is a suitable source of ascorbic acid for ruminants.

Figure 6.1 illustrates the correlation between plasma and muscle ascorbic acid levels in the animals receiving supplemental ascorbic acid. There was a significant correlation (P<0.05) coefficient between plasma and the levels of ascorbic acid of the biceps femoris. The correlation coefficient was $r^2$ =0.6843 (Figure 6.1). Ascorbyl-2-polyphosphate (Figure 6.2) additions raised the plasma levels of the animals and the amount the plasma was elevated was positively correlated to the increase in muscle ascorbic acid.

Neutrophil function can be enhanced in humans by the supplementation of dietary ascorbic acid (Bendich et al., 1986). Neutrophil ascorbic acid levels in humans is normally 24 ng/$10^6$ cells (Kutnink and Hawkes, 1987) which mimics closely the levels reported in Table 6.3 for bovine neutrophils. The data were quite variable between animals and there were not any differences (P>0.05) between groups at any stage of the trial. There are several explanations for the enhanced plasma levels not translating into increased neutrophil ascorbic acid levels. Firstly,

neutrophils actively transport ascorbic acid against a large concentration gradient, therefore, the cells can maintain a homeostatic level of the vitamin unless the host animal is deficient (Washko et al., 1989). It appears the trial animals were producing sufficient ascorbic acid to fulfil their metabolic requirements. Secondly, the amount of time needed to harvest and separate the cells may have been too long. Ascorbic acid is extremely labile and therefore, the time for processing may have masked any potential effects.

The absorption and post absorptive metabolic properties of iron, copper and zinc have been shown to be influenced by dietary ascorbic acid (Yen and Pond, 1981; Yen, 1984; Solomons and Viteri, 1982). Table 6.4 illustrates that the ascorbyl-2-polyphosphate did not alter the absorptive potentials of any of the trace minerals examined. The plasma levels of zinc, copper and iron were all within normal reported values for cattle (Puls, 1990).

## 6.4. CONCLUSIONS

Studies were performed to determine the suitability of ascorbyl-2-polyphosphate as a source of ascorbic acid for dairy heifers. After ascorbyl-2-polyphosphate was fed for 31 days at a rate of 80 g per head per day there was a significant increase in plasma levels of the test versus control animals, 4.56 and 3.58 mg/L respectively ($P<0.05$). Biopsies of the biceps femoris also showed increased ascorbic acid levels of the test group at $P= 0.08$ level of significance.

The plasma level of ascorbic acid in the test animals was positively correlated to the muscle levels ($P<0.05$). Ascorbyl-2-polyphosphate did not alter neutrophil ascorbic acid content or the plasma levels of copper, zinc or iron. Further studies would need to be conducted on the use of ascorbyl-2-polyphosphate to enhance meat quality and determine what, if any, effect that the compound may have on the immune system of ruminants.

**Table 6.1. Ascorbic acid plasma levels of dairy heifers fed ascorbyl-2-polyphosphate for 31 days[1].**

| | Plasma ascorbic acid levels[2] (mg/L) | | P-values |
|---|---|---|---|
| | Test (n=6) | Control (n=6) | |
| Before test | 3.72 ± 0.33 | 3.83 ± 0.35 | P>.05 |
| After test | 4.56 ± 0.58 | 3.58 ± 0.64 | P<.05 |

1 Ascorbic acid was fed at a rate of 20 grams per head per day

2 Mean ± Standard deviation

**Table 6.2. Ascorbic acid content of biceps femoris of dairy heifers fed ascorbyl-2-polyphosphate.**

| | Muscle ascorbic acid levels[1] (mg/Kg) | | P-values |
|---|---|---|---|
| | Test (n=6) | Control (n=6) | |
| Before treatment | 7.80 ± 1.30[2] | 8.10 ± 1.10 | P > .05 |
| After treatment | 10.11± 1.21 | 8.32 ± 1.66 | P = .08 |

1 Ascorbic acid was fed at a rate of 20 grams per head per day

± Standard deviation

**Table 6.3. Neutrophil ascorbic acid content of dairy heifers fed ascorbyl-2-polyphosphate.**

| | Neutrophil ascorbic acid levels[1] (ng/$10^6$cells | | P-values |
|---|---|---|---|
| | Test (n=6) | Control (n=6) | |
| Before test | 15.30 ± 4.60 | 18.20 ± 6.60 | P > .05 |
| After test | 16.60 ± 6.19 | 22 46 ± 6.40 | P > .05 |

1 Mean ± standard deviation

**Table 6.4. Zinc, copper and iron plasma levels of dairy heifers fed ascorbyl-2-polyphosphate (mg/L).**

| | Control (n = 6) | Test (n = 6) | P-values |
|---|---|---|---|
| Before Test | | | |
| Zinc | 1.80 ± 0.59[1] | 1.57 ± 0.26 | P > .05 |
| Copper | 1.04 ± 0.36 | 0.90 ± 0.14 | P > .05 |
| Iron | 1.17 ± 0.54 | 1.01 ± 0.07 | P > .05 |
| After Test | | | |
| Zinc | 2.14 ± 0.83 | 1.71 ± 0.25 | P > .05 |
| Copper | 1.19 ± 0.09 | 1.08 ± 0.13 | P > .05 |
| Iron | 1.26 ± 0.22 | 1.21 ± 0.10 | P > .05 |

1 Mean ± standard deviation

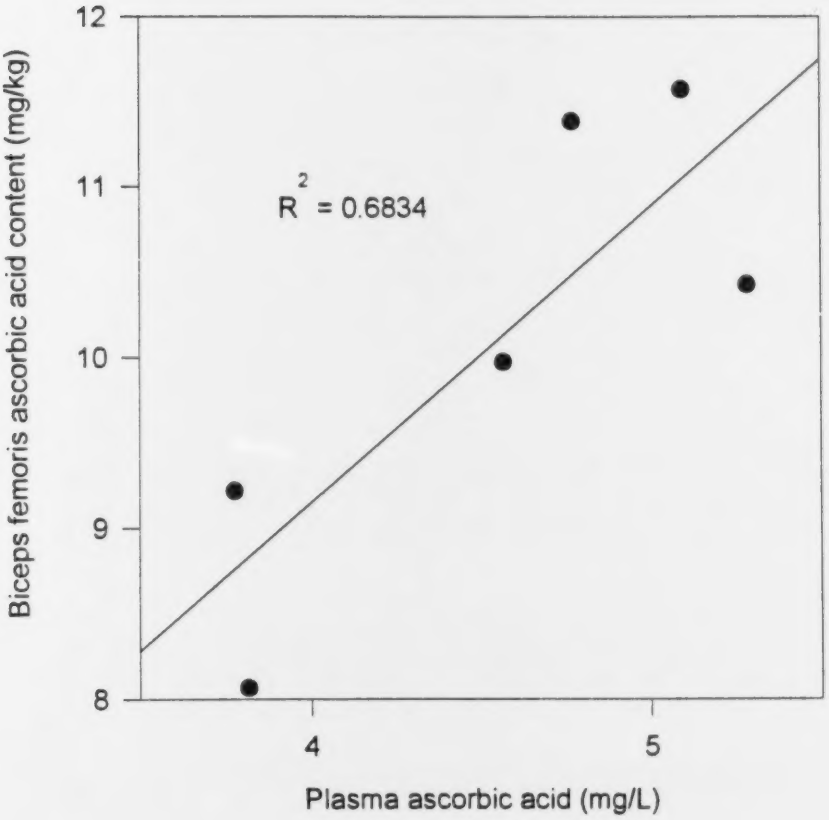
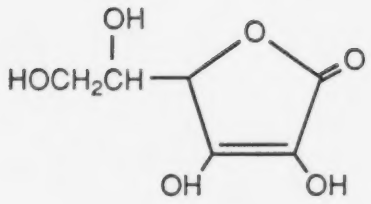
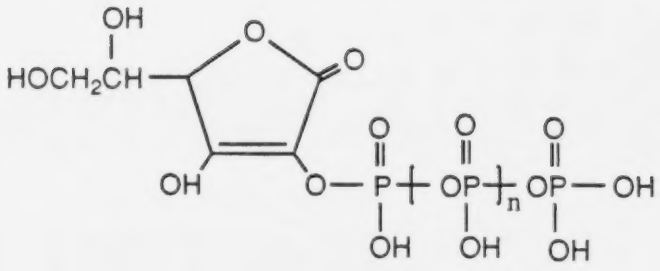



Figure 6.1: Ascorbic acid plasma levels versus levels of the vitamin in the biceps femoris




(A) Ascorbic acid




(B) Ascorbate-2-polyphosphate

Figure 6.2 Structures of ascorbic acid (A) and ascorbyl-2-polyphosphate (B)

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# CHAPTER 7

## Concluding Remarks

### 7.1. SUMMARY OF RESEARCH FINDINGS

Ascorbic acid is thought to have a role in the production and or regulation of corticosteroid production in the bovine adrenal gland. Stress and the associated rise in corticosteroids may reduce the circulating plasma levels of ascorbic acid. The effect of abomasal ascorbic acid infusion on plasma cortisol concentrations in lactating dairy cows, injected with ACTH to mimic stress, was investigated. It was determined that plasma cortisol and ascorbic acid were not altered ($P>0.05$) by the infusion of ascorbic acid into the abomasum of the treated dairy cows. There was however a trend which indicated that ascorbic acid can be absorbed by mature dairy cattle post rumen.

Researchers have noted that domestic animals that are experiencing stress often show reduced ascorbic acid levels. The reduced levels of circulating ascorbic acid is correlated to increased levels of plasma corticosteroids and a subsequent

reduction in the efficiency of the immune response. In cattle that have been injected with synthetic glucocorticoids the negative effect on neutrophil function could partially be alleviated by the injection of ascorbic acid. Unfortunately, injecting ascorbic acid can often lead to inflammation at the site of the injection. A study investigating the feasibility of orally administering ascorbic acid, in both the crystalline and polyphosphate form, was undertaken. Both forms of ascorbic acid were incubated in rumen fluid to determine the stability properties. Based on the reaction rate constants and the half-lives of both vitamin forms it can be concluded that ascorbyl-2-polyphosphate is a more rumen fluid stable form of ascorbic acid. Due to the stability of ascorby-2-polyphospate may prove useful as a source of orally administered vitamin C.

In addition to the orally administered vitamin C trial, the disappearance rate of ascorbic acid injected directly into the jugular vein of dairy cattle was also investigated. Ascorbic acid distributed itself according to a two compartment open model. The terminal half-life was determined to be 11.4 hr.

Stress has an impact on the immune system of animals therefore efficient techniques are required in order to assess the impact. The isolation of purified viable neutrophils from the whole blood of dairy cows was a critical step towards a better understanding as to how the innate disease fighting mechanisms of the cow might be enhanced. The procedures that were at hand for separating bovine neutrophils were time consuming and required relatively large quantities of blood.

Using Iscove's Modified Dulbecco's Medium layered onto Ficoll-Pacque and centrifugation, a erythrocyte, polymorphonuclear leukocyte combination was obtained. By selectively lysing the erythrocytes a relatively pure, viable polymorphonuclear leukocyte preparation was obtained.

Generally, it has been assumed that endogenously produced ascorbic acid was sufficient to meet the metabolic demands of ruminants. Lactating dairy cattle may be predisposed to subclinical ascorbic acid deficiency because of the demand for the precursor molecules of ascorbic acid production, glucose and galactose into milk. Studies were conducted to determine the suitability of ascorbyl-2-polyphosphate as a source of ascorbic acid for dairy heifers. After feeding the polyphosphate form of ascorbic acid for 31 days at a rate of 80 g per head per day there was a significant ($P<0.05$) increase in plasma levels of the test versus control animals. Biopsies of the biceps femoris also showed increased ascorbic acid levels of the test group at $P=0.08$ level of significance. The plasma level of ascorbic acid in the test animals was positively correlated to the muscle levels ($P<0.05$).

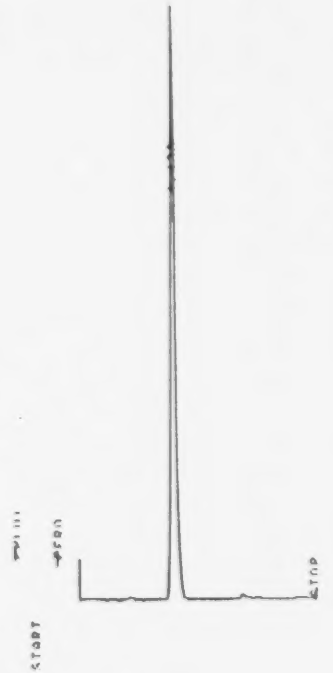
## 7.2. RECOMMENDATIONS FOR FUTURE WORK

The meat and dairy industries will continue to look for ways to cost effectively enhance their productivity and profitability through treatment. Ascorbic acid oral supplementation has potential as both a means of diminishing some of the impact of stress and a antioxidant in meat. It has been noted that direct injection of

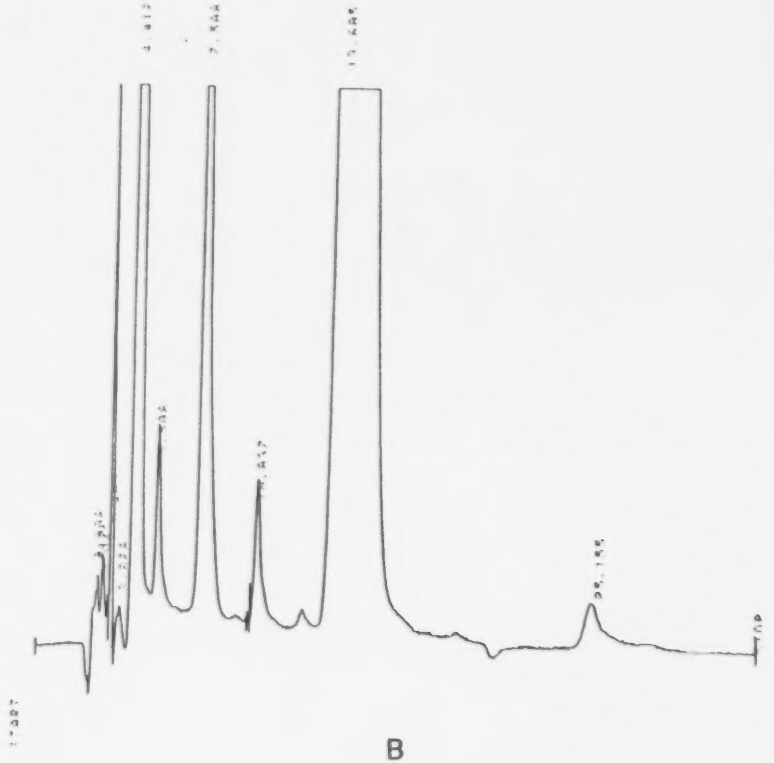
vitamin C can produce lesions and therefore trials further defining the kinetics of orally administered vitamin would be required. For example, ascorbyl-2-polyphosphate did not alter neutrophil ascorbic acid content or the plasma levels of copper, zinc or iron. Additional investigative work is required on the use of the particle forms of ascorbic acid to enhance meat quality and determine what, if any, effect that the compound may have on the immune system of ruminants.

## APPENDIX

High performance liquid chromatography of ascorbic acid.
A: Standard solution of ascorbic acid. B: Ascorbic acid in muscle samples. Column: OD 32 C18 (44250mm-5µm); detector: LC-4B Amperometric with a silver/silver chloride electrode; mobile phase: 5% Methanol in 0.1 mM $Na_2HPO_4$, 0.07 mM $EDTANa_2$, 0.15 mM $CH_3(CH_2)_7\ OSO_3Na$; pH 3.1.



A



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